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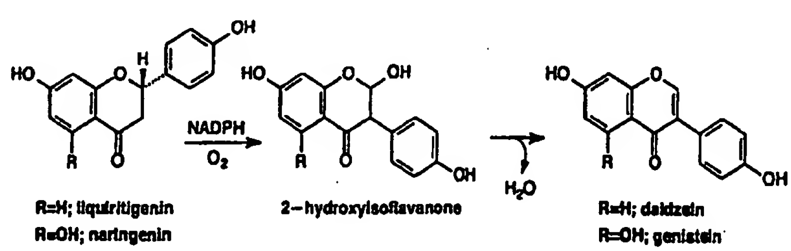

(57) Abstract

Soybean and *Medicago truncatula* CYP93C genes have been isolated which encode a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone. Plants can now be genetically engineered to produce isoflavones that provide potential human health benefits and increase disease resistance in plants. Isoflavones can now be produced in transgenic plants species in which isoflavones do not naturally occur, i.e., in species other than legumes. Alternatively, introducing infection-inducible isoflavonoid biosynthesis into non-legumes qualitatively complements these plants' phytoalexin defenses against microbial pathogens, whereas over-expression of the isoflavonoid pathway in legumes quantitatively increases this defense response. Finally, modifying the extend of production of isoflavonoids in legume roots positively impacts nodulation efficiency and therefore plant yield.

GENETIC MANIPULATION OF ISOFLAVONOIDS

TECHNICAL FIELD OF THE INVENTION

The invention relates to gene manipulation in plants.

BACKGROUND OF THE INVENTION

The flavonoids are a major class of phenylpropanoid-derived plant natural products. Their fifteen carbon (C_6-C_3-C_6) backbone can be arranged as a 1,3-diphenylpropane skeleton (flavonoid nucleus) or as a 1,2-diphenylpropane skeleton (isoflavonoid nucleus). Although 1,3-diphenylpropane flavonoid derivatives are almost ubiquitous among terrestrial plants, the 1,2-diphenylpropane isoflavonoids are restricted primarily to the Leguminosae, although they occur rarely in other families such as the Apocynaceae, Pinaceae, Compositae, and Moraceae (Tahara, S. and R. K. Ibrahim, 1995, "Prenylated isoflavonoids - an update," *Phytochemistry* 38: 1073-1094).

The limited taxonomic distribution of the isoflavonoids is directly related to the occurrence of the enzyme complex isoflavone synthase (IFS), which catalyzes the aryl migration reaction leading to the formation of an isoflavone from a flavanone. While flavanones are ubiquitous in higher plants, the IFS reaction, which is a two-step process specific for isoflavonoid biosynthesis (Kochs, G. and H. Grisebach, 1986, "Enzymic synthesis of isoflavones," *European J Biochem* 155: 311-318), is limited to the Leguminosae and the other diverse taxa in which isoflavonoids are occasionally found.

The presence of isoflavonoids provides several advantages to plants. One such advantage is provided by the function of isoflavonoids as antimicrobial phytoalexins in plant-microbe interactions. For example, the simple isoflavones daidzein and genistein act as initial precursors in the biosynthesis of various antimicrobial isoflavonoid phytoalexins in a wide variety of legumes (Dixon, R. A. and N. L. Paiva, 1995, "Stress-induced phenylpropanoid metabolism," *Plant Cell* 7: 1085-1097). Isoflavonoid compounds have been shown to accumulate in infected plant cells to

levels known to be antimicrobial *in vitro*. The temporal, spatial and quantitative aspects of accumulation are consistent with a role for these compounds in disease resistance (Rahe, J. E., 1973, "Occurrence and levels of the phytoalexin phaseollin in relation to delimitation at sites of infection of *Phaseolus vulgaris* by *Colletotrichum lindemuthianum*," *Canadian J Botany* 51: 2423-2430; Hadwiger, L. A. and D. M. Webster, 1984, "Phytoalexin production in five cultivars of pea differentially resistant to three races of *Pseudomonas syringae* pv. *pisi*," *Phytopathology* 74: 1312-1314; Long, et al., 1985, "Further studies on the relationship between glyceollin accumulation and the resistance of soybean leaves to *Pseudomonas syringae* pv. *glycinea*," *Phytopathology* 75: 235-239; Bhattacharyya, M. K. and E. W. B. Ward, 1987, "Biosynthesis and metabolism of glyceollin I in soybean hypocotyls following wounding or inoculation with *Phytophthora megasperma* f. sp. *glycinea*," *Physiol and Mol Plant Pathology* 31: 387-405). Moreover, it has been reported that many plant pathogens are much more sensitive to phytoalexins of non-host species than they are to the phytoalexins of their natural hosts, because they can often detoxify the host's phytoalexins. (VanEtten, et al., 1989, "Phytoalexin detoxification: importance for pathogenicity and practical implications," *An Rev Phytopathology* 27: 143-164).

Isoflavonoids also function in plant-microbe interactions in the establishment of bacterial or fungal symbioses with plants. Isoflavonoids have been reported to regulate bacterial nodulation genes, acting as a major *nod* gene inducer (Kosslak, et al., 1987, "Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*," *Proc Natl Acad Sci USA* 84: 7428-7432) and/or transcription activator (Dakora, et al., 1993, "Common bean root exudates contain elevated levels of daidzein and coumestrol in response to *Rhizobium* inoculation," *Mol Plant-Microbe Interact* 6: 665-668). Isoflavonoids have also been shown to have a role on the establishment of the symbiotic vesicular arbuscular mycorrhizal (VAM) association of the fungus *Glomus* with legume roots. (Kape, et al., 1992, "Legume root metabolites and VA-mycorrhiza development," *J Plant Physiol* 141: 54-60). Xie et al have reported that the isoflavonoids coumestrol, daidzein and genistein have small but significant stimulatory effects on the degree of mycorrhizal colonization of soybean, and that one effect of isoflavonoids on the

soybean mycorrhizal symbiosis could be via induction of nodulation factors from co-colonizing Rhizobia, since nod-factors have also been shown to stimulate fungal colonization (Xie, et al., 1995, "Rhizobial nodulation factors stimulate mycorrhizal colonization of nodulating and nonnodulating soybeans," *Plant Physiology* 108: 1519-1525).

In addition to the advantages that the presence of isoflavonoids confers to plants, a significant body of evidence indicates that dietary consumption of isoflavonoids can provide benefits to human health. Dietary isoflavones have been ascribed strong cancer chemopreventative activity in humans, and display a range of pharmacological activities suggestive of various other health promoting effects, including phytoestrogen activity as both estrogenic and anti-estrogenic agents (Coward, et al., 1993, "Genistein, daidzein, and their -glycoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets," *J Agricultural and Food Chemistry* 41: 1961-1967; Martin, et al., 1996, "Interactions between phytoestrogens and human sex steroid binding protein," *Life Sciences* 58: 429-436); anticancer effects associated with phytoestrogenic activity (Lee, et al., 1991, "Dietary effects on breast-cancer risk in Singapore," *Lancet* 337: 1197-1200; Adlercreutz, et al., 1991, "Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet," *Am J Clin Nutr* 54: 1093-1100); anticancer effects associated with inhibition of several enzymes including DNA topoisomerase and tyrosine protein kinase (Akiyama, et al., 1987, "Genistein, a specific inhibitor of tyrosine-specific protein kinases," *J Biol Chem* 262: 5592-559; Uckun, et al., 1995, "Biotherapy of B-cell precursor leukemia by targeting genistein to CD19-associated tyrosine kinases," *Science* 267: 886-891);suppression of alcohol consumption (Keung, W. M. and B. L. Vallee, 1993, "Daidzin: A potent, selective inhibitor of human mitochondrial aldehyde dehydrogenase," *Proc Natl Acad Sci USA* 90: 1247-1251; Keung, et al., 1995, "Daidzin suppresses ethanol consumption by Syrian golden hamsters without blocking acetaldehyde metabolism," *Proc Natl Acad Sci USA* 92: 8990-8993); antioxidant activity (Arora, et al., 1998, "Antioxidant activities of isoflavones and their biological metabolites in a lipsomal system," *Arch Biochem Biophys* 356: 133-141; Tikkanen, et

al., 1998, "Effect of soybean phytoestrogen intake on low density lipoprotein oxidation resistance," *Proc Natl Acad Sci USA* 95: 3106-3110); effects on calcium metabolism, some of which may be linked to protective effects against osteoporosis (Tomonaga, et al., 1992, "Isoflavonoids, genistein, PSI-tectorigenin, and orobol, increase cytoplasmic free calcium in isolated rat hepatocytes," *Biochem Biophys Res Com* 182: 894-899; Draper, et al., 1997, "Phytoestrogens reduce bone loss and bone resorption in oophorectomized rats," *J Nutr* 127: 1795-1799); and cardiovascular effects (Wagner, et al., 1997, "Dietary soy protein and estrogen replacement therapy improve cardiovascular risk factors and decrease aortic cholesteryl ester content in ovariectomized cynomolgus monkeys," *Metabolism - Clinical and Experimental* 46: 698-705).

At present, the only dietary sources of isoflavonoids for humans are certain legumes such as soybean or chickpea. The development of methods to genetically manipulate isoflavonoids in plants, either to widen the source of dietary isoflavonoids for humans, or to exploit the biological activities of isoflavonoids for plant protection and improvement, is wholly dependent on the availability of cloned genes encoding the various enzymes of isoflavonoid biosynthesis. Of these, the isoflavone synthase (IFS) complex constitutes the first committed reactions, and as such represents the means to introduce isoflavonoids into plants that do not possess the pathway.

In 1984, Hagmann and Grisebach provided the first evidence for the enzymatic conversion of flavanone to isoflavone (the IFS reaction) in a cell free system (Hagmann, M. and H. Grisebach, 1984, "Enzymatic rearrangement of flavanone to isoflavone," *FEBS Letters* 175: 199-202). They demonstrated that microsomes from elicitor-treated soybean cell suspension cultures could catalyze the conversion of 2(*S*)-naringenin to genistein, or of 2(*S*)-liquiritigenin to daidzein, in the presence of NADPH. The crude microsomal enzyme preparation, which was stable at -70°C but had a half-life of only 10 minutes at room temperature, was absolutely dependent on NADPH and molecular oxygen. It was subsequently shown that the reaction proceeded in two steps. The flavanone was converted in a cytochrome P450-catalyzed reaction requiring NADPH and O_2 to the corresponding 2-hydroxyisoflavanone. This

relatively unstable compound, which could, however, be identified by mass spectrometric analysis, then underwent dehydration to yield the isoflavone. The dehydration reaction appeared to be catalyzed by an enzyme present predominantly in the cytoplasmic supernatant, although it was not possible to remove all this activity from the microsomes. The corresponding 2-hydroxyisoflavanone spontaneously converted to genistein, for example, in methanol at room temperature. Kinetic analysis indicated that the 2-hydroxyisoflavanone was formed prior to genistein, consistent with its being an intermediate in isoflavone formation. (Kochs, G. and H. Grisebach, 1986, "Enzymic synthesis of isoflavones," *European J Biochem* 155: 311-318).

Involvement of cytochrome P450 in the 2-hydroxyisoflavanone synthase reaction was confirmed by inhibition by CO, replacing O_2 with N_2, and examining the effects of a range of known P450 inhibitors of which ancymidol was the most effective. The enzyme co-migrated with the endoplasmic reticulum markers cinnamate 4-hydroxylase (another cytochrome P450) and cytochrome b5 reductase on Percoll gradients. The enzyme is stereoselective, and (2*R*)-naringenin is not a substrate. (Kochs, G. and H. Grisebach, 1986, "Enzymic synthesis of isoflavones," *European J Biochem* 155: 311-318).

The origin of the 2-hydroxyl group was determined from studies on the IFS present in microsomes from elicited cell cultures of *Pueraria lobata*. ^{18}O from $^{18}O_2$ was incorporated into the 2-hydroxyl group, resulting in a 2-hydroxyisoflavanone with molecular ion shifted by two mass units, whereas there was no corresponding shift in the molecular ion of daidzein, consistent with the subsequent dehydration reaction (Hashim, et al., 1990, "Reaction mechanism of oxidative rearrangement of flavanone in isoflavone biosynthesis," *FEBS Letters* 271: 219-222). The currently accepted model for the reaction pathway of IFS as illustrated in Fig. 1, therefore, involves P450-catalyzed hydroxylation coupled to aryl migration, a reaction with mechanistic similarities to the well described proton migration mechanism of some P450 reactions (Hakamatsuka, et al., 1991, "P-450-dependent oxidative rearrangement in isoflavone

biosynthesis: reconstitution of P-450 and NADPH:P450 reductase," *Tetrahedron* 47: 5969-5978).

Currently, there have been no reports on purification to homogeneity or molecular cloning of the cytochrome P450 of the IFS complex because of the extreme lability of the enzyme. The 2-hydroxyisoflavanone synthase cytochrome P450 from *Pueraria* has been solubilized with Triton X-100, and partially purified by DEAE-Sepharose chromatography; the enzymatic reaction could be reconstituted by addition of NADPH cytochrome P450 reductase that separated from the hydroxylase on the ion exchange column (Hakamatsuka, et al., 1991, *Tetrahedron* 47: 5969-5978). A 2-hydroxyisoflavanone dehydratase has been purified from elicitor-treated *P. lobata* cells, and has been shown to be a soluble monomeric enzyme of subunit Mr 38,000 (Hakamatsuka, et al., 1998, "Purification of 2-hydroxyisoflavanone dehydratase from the cell cultures of *Pueraria lobata*," *Phytochemistry* 49: 497-505). It is not yet clear whether this enzyme physically associates with the P450 hydroxylase catalyzing the aryl migration, or even whether this activity is essential for isoflavone formation *in planta* in view of the spontaneous conversion of 2-hydroxyisoflavanone to isoflavone.

Flavanone is a potential substrate for more than one type of hydroxylation reaction at the 2-position. Thus, elicitor-treated cell cultures of alfalfa and *Glycyrrhiza echinata* have been shown to accumulate the dibenzoylmethane licodione (Kirikae, et al., 1993, "Biosynthesis of a dibenzoylmethane, licodione, in cultured alfalfa cells induced by yeast extract," *Biosci Biotech Biochem* 57: 1353-1354). Licodione synthase is, by classical criteria, a cytochrome P450, the activity of which is induced by yeast elicitor in *Glycyrrhiza* cells (Otani, et al., 1994, "Licodione synthase, a cytochrome P450 monooxygenase catalyzing 2-hydroxylation of 5-deoxyflavanone, in cultured *Glycyrrhiza echinata* L. cells," *Plant Physiol* 105: 1427-1432). The reaction it catalyzes involves 2-hydroxylation of flavanone followed by hemiacetal opening instead of aryl migration, and the reaction was thought to have mechanistic similarities to the flavone synthase II enzyme previously characterized from soybean (Kochs, G. and H. Grisebach, 1987, "Induction and characterization of a NADPH-dependent flavone synthase from cell cultures of soybean," *Z. Naturforsch* 42C: 343-

348). A gene encoding the flavone synthase II/licodione synthase from *Glycyrrhiza* has been cloned (Akashi, et al., 1998, "Identification of a cytochrome P450 cDNA encoding (2S)-flavanone 2-hydroxylase of licorice *(Glycyrrhiza echinata* L.: *Fabaceae)* which represents licodione synthase and flavone synthase II," *FEBS Letters* 431: 287-290), and a different cytochrome P450 gene encoding flavone synthase II has recently been cloned from *Gerbera hybrida* (Martens, S. and G. Forkmann, "Cloning and expression of flavone synthase II from Gerbera hybrids," *Plant J*20: 611-618).

Although the reactions catalyzed by IFS are critical for the formation of all isoflavonoids in plants, there have been no previous reports of the isolation of genes encoding components of isoflavone synthase, although genes encoding most of the other enzymes of the isoflavonoid pathway, including downstream enzymes converting simple isoflavones to antimicrobial phytoalexins, have been characterized (Dixon, et al., 1995, "The isoflavonoid phytoalexin pathway: from enzymes to genes to transcription factors," *Physiologia Plantarum* 93: 385-392). Thus, the unavailability of isoflavone synthase genes has made it heretofore impossible to utilize the downstream genes for regulating isoflavonoid concentrations in legumes and other plants that do have the isoflavonoid pathway, or for engineering antimicrobial and pharmacologically active isoflavonoids in transgenic plants of species that do not have the isoflavonoid pathway.

Genes encoding the enzyme catalyzing the first step of the isoflavone synthase reaction have now been isolated and purified from soybean and *Medicago truncatula* (barrel medic).

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts the currently accepted model for the reaction pathway of IFS wherein the flavanone is converted in a cytochrome P450-catalyzed reaction requiring NADPH and O_2 to the corresponding 2-hydroxyisoflavanone which then undergoes dehydration to yield the isoflavone.

Fig. 2 depicts the nucleotide sequence of soybean CYP93Clv2.

Fig. 3 depicts the amino acid sequence of soybean CYP93C1v2 compared to licorice CYP93B1.

Fig. 4 depicts the nucleotide sequence of *Medicago truncatula* mtIFSE3.

Fig. 5 depicts the amino acid sequence of *Medicago truncatula* mtIFSE3 compared to soybean CYP93C1v2.

Fig. 6A and Fig. 6B depict HPLC traces of extracts from pooled tissues (leaves, shoots, flowers) of *Arabidopsis thaliana* ecotype Columbia harboring an empty tDNA vector (Fig. 6A) and *Arabidopsis thaliana* ecotype Columbia harboring the soybean CYP93C1v2 cDNA sequence (Fig. 6B). The empty vector transformed line contains a number of flavonol glycosides and other phenolic compounds that are also present in the CYP93C1v2 transformed line. These compounds were identified as (a) rhamnose (Rha)- glucose (Glc)- quercetin (Q), (b) uncharacterized conjugate of Q, (c) Rha-Glc-Rha-Kaempferol (K), (d) Glu-Rha-Q, (e) Rha-Rha-Q, (f) Glc-Rha-K, (g) sinapic acid, (h) Rha-Rha-K. Three additional compounds were observed in the CYP93C1v2 transformed line (Fig. 6B), and labeled "1," "2" and "3." Fig. 6C depicts a total ion chromatogram of partially purified peaks 2 and 3, and the insets show the specific ions generated from these compounds. Peak 2 has a parental molecular mass ion of 579.5 consistent with genistein conjugated to a glucose-rhamnose disaccharide, and two further mass ions of 417.5 and 271.3, representing Rha-genistein and free genistein, respectively. Peak 3, which has a parental molecular ion of mass 417.5, is thereby identified as Rha-genistein.

Fig. 7A and Fig. 7B depict HPLC traces of the same extracts as shown in Fig. 6A (empty-vector transformed) and Fig. 6B, (CYP93C1v2 transformed), but following digestion with β-glucosidase. Peaks 2 and 3 remained at the same retention time as in Fig. 6A and 6B. However, Peak 1 disappeared, and was replaced with a new Peak 4 of much later retention time. Fig. 7C shows the total ion chromatograph of purified Peak 4, and the inset shows the parental molecular ion, with mass of 271.2, consistent with Peak 4 being free genistein. Fig. 7D shows a total ion chromatograph, and the parental molecular ion, of an authentic sample of genistein.

Fig. 8A , 8B, 8C and 8D are high performance liquid chromatography (HPLC) chromatograms depicting the presence of new peaks at RT 29.96 and 37.7 min representing the presence of the isoflavone daidzein formed from the flavanone liquiritigenin, or the isoflavone genistein formed from the flavanone naringenin, in insect cell microsomes expressing CYP93C1v2. Fig. 8A depicts the presence of NADPH during incubation with liquiritigenin. Fig. 8B depicts the absence of NADPH during incubation with liquiritigenin. Fig. 8C depicts the presence of NADPH during incubation with naringenin. Fig. 8D depicts the lack of a reaction when soybean CYP93E expressed in insect cells is incubated with liquiritigenin in the presence of NADPH.

Fig. 9A and Fig. 9B are mass spectra of BSTFA (*N,O*-bis(trimethylsilyl) trifluoroacetamide) derivatives. Fig. 9A depicts the mass spectrum of the BSTFA derivative of the product of the reaction catalyzed by CYP93C1v2 in insect cells using liquiritigenin as substrate, and Fig. 9B shows the mass spectrum of the BSTFA derivative of an authentic sample of daidzein .

SUMMARY OF THE INVENTION

In one aspect, the invention is a method for introducing into a naturally non-isoflavonoid-producing plant species the enzyme catalyzing the aryl migration of a flavanone to form an isoflavanone intermediate or an isoflavone, comprising introducing a DNA segment encoding the enzyme into the plant to form a transgenic plant, wherein the transgenic plant expresses the DNA segment under the control of a suitable constitutive or inducible promoter when the transgenic plant is exposed to conditions which permit expression. The DNA segment can comprise isolated genomic DNA or recombinant DNA. Preferably, the DNA segment is a CYP93C gene. An exemplary DNA segment from a soybean CYP93C gene consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another preferred DNA segment comprises a *Medicago truncatula* homolog of a CYP93C gene, more preferably, the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4. Plants transformed by this method may also preferably express chalcone synthase,

chalcone reductase, and chalcone isomerase genes to cause in vivo formation of daidzein or a daidzein derivative, and the chalcone synthase, chalcone reductase, and chalcone isomerase genes may also be transgenes. Plants transformed by this method may also preferably further comprise downstream genes, for example, isoflavone *O*-methyltransferase, isoflavone 2'-hydroxylase, isoflavone reductase, and vestitone reductase, to metabolize a formed isoflavone to biologically active isoflavonoid derivatives or conjugates. The plant can comprise isoflavone 4'-*O*-methyl-transferase to cause formation of biochanin A or a biochanin A derivative from the isoflavanone intermediate. An exemplary flavanone substrate for this transformation method is liquiritigenin and/or naringenin.

In another aspect, the present invention is a method for increasing the level of isoflavonoid compounds in naturally isoflavonoid-producing plants comprising introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to yield an isoflavonoid to form a transgenic plant, wherein the transgenic plant expresses the DNA segment under the control of a suitable constitutive or inducible promoter when the transgenic plant is exposed to conditions which permit expression. With this method, the resulting isoflavonoid can be an isoflavanone intermediate, an isoflavone, an isoflavone derivative, and an isoflavone conjugate. The DNA segment can comprise isolated genomic DNA or recombinant DNA. Preferably, the DNA segment is a CYP93C gene. An exemplary DNA segment from a soybean CYP93C gene consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another preferred DNA segment comprises a *Medicago truncatula* homolog of a CYP93C gene, more preferably, the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4. An exemplary flavanone substrate for this transformation method is liquiritigenin and/or naringenin.

In another aspect, the invention is a method for synthesizing an isoflavanone intermediate or an isoflavone from a flavanone by expressing a recombinant CYP93C gene segment in a suitable bacterial, fungal, algal, or insect cell system. An exemplary gene segment consists essentially of the sequence from about nucleotide 36

to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another exemplary gene segment consists essentially of the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

In another aspect, the invention is a method of reducing the levels of isoflavonoid compounds in a naturally isoflavonoid-producing plant comprising introducing and expressing an antisense or gene silencing construct that contains an intact CYP93C gene or segments thereof into the plant. An exemplary gene consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another exemplary gene consists essentially of the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

In another aspect, the invention is a naturally non-isoflavonoid-producing plant cell transformed by introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to form an isoflavanone intermediate or an isoflavone, wherein the transformed plant cell expresses the DNA segment under the control of a suitable constitutive or inducible promoter when exposed to conditions which permit expression. The DNA segment can comprise isolated genomic DNA or recombinant DNA. Preferably, the DNA segment is a CYP93C gene. An exemplary DNA segment from a soybean CYP93C gene consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another preferred DNA segment comprises a *Medicago truncatula* homolog of a CYP93C gene, more preferably, the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4. Plants transformed by this method may also preferably express chalcone synthase, chalcone reductase, and chalcone isomerase genes to cause in vivo formation of daidzein or a daidzein derivative, and the chalcone synthase, chalcone reductase, and chalcone isomerase genes may also be transgenes. Plants transformed by this method may also preferably further comprise downstream genes, for example, isoflavone *O*-methyltransferase, isoflavone 2'-hydroxylase, isoflavone reductase, and vestitone reductase, to metabolize a formed isoflavanone intermediate to biologically active isoflavonoid

derivatives or conjugates. The plant can comprise isoflavone 4'-*O*-methyl-transferase to cause formation of biochanin A or a biochanin A derivative from the isoflavanone intermediate.

In another aspect, the invention is a naturally isoflavonoid-producing plant cell transformed by introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to yield an isoflavonoid to form a transformed plant cell, wherein the transformed plant cell expresses the DNA segment under the control of a suitable constitutive or inducible promoter when exposed to conditions which permit expression. With this method, the resulting isoflavonoid can be an isoflavanone intermediate, an isoflavone, an isoflavone derivative, and an isoflavone conjugate. The DNA segment can comprise isolated genomic DNA or recombinant DNA. Preferably, the DNA segment is a CYP93C gene. An exemplary DNA segment from a soybean CYP93C gene consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another preferred DNA segment comprises a *Medicago truncatula* homolog of a CYP93C gene, more preferably, the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

In another aspect, the invention is a transgenic plant cell having reduced levels of isoflavonoid compounds, the plant cell transformed by introducing an antisense or gene silencing construct that contains an intact CYP93C gene or segments thereof into the plant cell. An exemplary gene consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another exemplary gene consists essentially of the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

In another aspect, the invention is an isolated gene or DNA segment comprising a portion which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the portion consists essentially of about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. An exemplary gene is the soybean gene encoding the enzyme catalyzing the aryl migration of liquiritigenin. Another

exemplary gene is the soybean gene encoding the enzyme catalyzing the aryl migration of naringenin.

In another aspect, the invention is a protein encoded by a portion of an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the portion consists essentially of about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

In another aspect, the invention is an isolated gene or DNA segment comprising a portion which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the portion is a *Medicago truncatula* homolog of a CYP93C gene. An exemplary gene or DNA segment consists essentially of about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4. An exemplary gene is the *Medicago truncatula* gene encoding the enzyme catalyzing the aryl migration of liquiritigenin. Another exemplary gene is the *Medicago truncatula* gene encoding the enzyme catalyzing the aryl migration of naringenin.

In another aspect, the invention is a protein encoded by a portion of an isolated gene or a DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the portion is a *Medicago truncatula* homolog of a CYP93C gene.

In yet another aspect, the invention is a food comprising edible transgenic plant material capable of being ingested for its nutritional value, wherein the transgenic plant has been transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, and wherein the transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a food comprising at least one isoflavonoid, wherein the isoflavonoid is isolated from a transgenic plant transformed

with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, and wherein the transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a composition comprising at least a portion of a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment, and wherein the composition is suitable for ingestion as a food stuff, a nutritional supplement, an animal feed supplement, or a nutraceutical.

In yet another aspect, the invention is a composition comprising an isoflavonoid suitable for administration as a food stuff, a nutritional supplement, an animal feed supplement, a nutraceutical, or a pharmaceutical, wherein the isoflavonoid is isolated from at least a portion of a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, and wherein the transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a method of increasing the nutritional value of a plant by transforming the plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a method of using a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment, to provide a nutraceutical benefit to a human or animal administered the isoflavonoid. The isoflavonoid can be administered by ingestion of at least a portion of the plant. The isoflavonoid can also be administered by ingestion of a composition comprising an isoflavonoid isolated from the plant.

In yet another aspect, the invention is a method of using an isoflavonoid isolated from a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment, to provide a pharmaceutical benefit to a patient administered the isoflavonoid.

In yet another aspect, the invention is a method of increasing disease resistance in a plant by transforming the plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a method of increasing nodulation efficiency of a leguminous plant by transforming the plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid released from the roots

when compared to the level of the isoflavonoid released from the roots of plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a transgenic leguminous plant exhibiting increased nodulation efficiency transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid released from the roots when compared to the level of the isoflavonoid released from the roots of plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a method of increasing bacterial or fungal symbiosis in a plant by transforming the plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a transgenic plant exhibiting increased bacterial or fungal symbiosis transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the recombinant DNA sequence.

In yet another aspect, the invention is seed from a transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the recombinant DNA sequence.

In yet another aspect, the invention is progeny from a transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the recombinant DNA sequence.

In yet another aspect, the invention is progeny from seed of a transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the recombinant DNA sequence.

In yet another aspect, the invention is use of a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment, for the preparation of a nutraceutical preparation for achieving a nutritional effect.

In yet another aspect, the invention is use of a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid

when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment, for the preparation of a pharmaceutical preparation for achieving a therapeutic effect.

DETAILED DESCRIPTION

One aspect of the present invention is an isolated gene which encodes the first step of the isoflavone synthase reaction: a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavone. Genes and corresponding cDNA of the soybean or *Medicago truncatula* CYP93 family have been isolated. The enzymes encoded by the genes of the present invention are isoflavone synthases (IFS) and can catalyze the aryl migration of a flavanone to yield an isoflavone either directly or through the intermediacy of a 2-hydroxyisoflavanone. One isolated soybean gene is classified as *CYP93C1v2*.

Cytochrome P450 enzymes belong to a large superfamily of enzymes that are abundant in every living organism. The P450 nomenclature committee has determined that each P450 should carry a "CYP" designation and arbitrarily divided the superfamily into families (alphabetical designation), subfamilies (numerical designation) and allelic variants ("v" plus numerical designation) based on amino acid identity of >40%, >55%, and >97%, respectively (Nelson, et al. 1993. "The P450 superfamily update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature," *DNA Cell Biol* 12:1). Thus, CYP93C1v2 is a variant of the first described P450 belonging to the third subfamily (C) of the ninety-third P450 family.

Utilizing the procedures presented herein, any plant known to produce isoflavonoids may also serve as sources of suitable DNA, or coding sequences may be synthesized in vitro based on the sequences for the IFS genes of the present invention. CYP93 family members can also be obtained from other plant species by polymerase chain reaction amplification methods known to those skilled in the art, using primer sequences corresponding to regions of nucleotide conservation between CYP93 family members. Furthermore, the genes of the present invention are defined by their

catalytic activity: the aryl migration of a flavanone to yield an isoflavone. The gene sequences presented as SEQ ID NO:1 and SEQ ID NO:4 are exemplary, and it is understood that modifications to these genes which do not alter the catalytic activity of its encoded protein fall within the scope of the present invention. While a preferred IFS gene contains the entire open reading frame, portions of or the entire 5' and 3' untranslated regions as well as portions of the vector sequence can also be present. With the isolation and functional identification of these isoflavone synthase (IFS) genes that encode the first key step in isoflavone formation, the aryl migration reaction, it is now possible to introduce the isoflavonoid pathway into all plant species, including those that do not naturally possess this pathway.

Another aspect of the present invention is a genetically modified plant which has been transformed with a gene of the present invention. For example, when the *CYP93C1v2* gene is transferred into the model plant *Arabidopsis thaliana*, which does not naturally produce isoflavonoids, the isoflavone genistein accumulates as a series of glycoconjugates (Example 1). This demonstrates that the genes of the present invention can be genetically engineered into plants which do not naturally contain the isoflavonoid pathway, and the transgenic plants can then produce isoflavonoids, resulting in plants with improved disease resistance and/or value added health benefits for humans. In the present invention, unless otherwise stated, as used herein, the term "plant" or "progeny" includes plant parts, plant tissue, plant cells, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, plant calli, plant clumps, explants, plant cells that are intact in plants, or parts of plants, such as embryos, pollen, ovules, flowers, capsules, stems, leaves, seeds, roots, root tips, and the like. Furthermore, the present invention includes the IFS genes expressed in various parts of the plant, e.g., in aerial portions of the plant useful for increasing disease resistance or production of health promoting isoflavonoid nutraceuticals, in seeds useful for increasing levels of isoflavones and their conjugates, or in roots useful for increasing disease resistance or production of nodulation gene inducing isoflavones.

In another aspect, the present invention is a method of improving disease resistance and a transgenic plant with increased disease resistance. By transforming a plant which does not naturally make isoflavones with an IFS gene of the present invention, disease resistance can be genetically engineered into the plant by providing the necessary enzyme to convert its natural flavanones into isoflavonoids. The introduction and subsequent expression of an IFS gene of the present invention into a crop species which naturally possesses the isoflavonoid pathway results in increased levels of the isoflavonoid defense compounds.

In another aspect, the present invention is a method of increasing levels of isoflavonoids that might be beneficial to the establishment of bacterial or fungal symbioses with plants and a transgenic plant with an increased capacity for symbiotic association with bacteria or fungi. Bacterial nodulation can be stimulated in transgenic leguminous plants by expression of an IFS gene of the present invention and decreased by expression of antisense constructs or constructs designed to promote gene silencing that contain an intact IFS gene or segments thereof. Mycorrhizal colonization of leguminous plants can also be increased through the introduction and expression of an IFS gene of the present invention.

In yet another aspect, the present invention is a method of producing isoflavonoid compounds in plants or any other organism to be used in nutraceuticals or pharmaceuticals to confer human or animal health benefits. Edible transgenic plants high in isoflavonoids can be utilized as food for humans and animals. Edible compositions high in isoflavonoids can also be made by incorporation of the transgenic plants or plant materials, or by incorporation of isoflavonoids isolated from the transgenic plants. Compositions useful for administration as a food stuff, a nutritional supplement, an animal feed supplement, a nutraceutical, or a pharmaceutical can be made by incorporation of the transgenic plants or plant materials, or by incorporation of isoflavonoids isolated from the transgenic plants. The nutritional value of a plant can be increased by transforming the plant with an IFS gene of the present invention and, as a result, accumulating high amounts of isoflavonoids in the plant.

The soybean IFS gene of the present invention was isolated and purified according to the detailed procedures outlined in Example 2. The DNA sequence is shown in SEQ ID NO:1 and Fig. 2, and the encoded protein sequence of the isolated soybean CYP93C clone is shown in SEQ ID NO:2 and Fig. 3. For comparison, Fig. 3 also shows the protein sequence alignment between the isolated CYP93C clone (SEQ ID NO:2) and CYP93B1 (SEQ ID NO:3), the licorice licodione synthase.

The DNA and protein sequences of the soybean CYP93C1 open reading frame were deposited in the Genbank data base under accession # AF022462. The deposition was made by Siminszky, Dewey and Corbin, and the sequence described as representing a gene induced in soybean in response to herbicide safeners. However, the function of the gene was not known and there was no understanding that it could be involved in isoflavonoid biosynthesis at the time the deposit was made (Siminszky, B., Corbin, F.T., Ward, E.R., Fleischmann, T.J. and Dewey, R.E. ,1999, "Expression of a soybean cytochrome P450 monooxygenase cDNA in yeast and tobacco enhances the metabolism of phenylurea herbicides." *Proc. Natl. Acad. Sci. USA* 96: 1750-1755). The sequence of the clone characterized herein differs from CYP93C1 in three nucleotide substitutions in the open reading frame that change proline 140 to leucine, threonine 156 to isoleucine, and glutamate 295 to lysine. Thus, the soybean gene identified herein has been classified as CYP93C1v2.

The cDNA insert from CYP93C1v2 was used to probe 240,000 phage plaques from a *Medicago truncatula* root cDNA library (van Buuren, M.L., I.E. Maldonado-Mendoza, A.T. Trieu, L.A. Blaylock, and M.J. Harrison, 1999, "Novel genes induced during an arbuscular mycorrhizal (AM) symbiosis formed between *Medicago truncatula* and *Glomus versiforme*," *Mol. Plant-Microbe Interact.* 12, 171-181). Five positive plaques were purified, in vivo excised, and sequenced. A full length clone designated mtIFSE3 was completely sequenced on both strands, and shown to encode the *Medicago truncatula* homolog of soybean CYP93C1. The nucleotide sequence of mtIFSE3 is shown in SEQ ID NO:4 and Fig. 4, and the protein sequence, in SEQ ID NO:5. An alignment between the protein sequences of mtIFSE3 and CYP93C1v2 is shown in Fig. 5.

An IFS gene of the soybean or *Medicago truncatula* CYP93C subfamily or corresponding cDNA sequence, the open reading frame of which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavone, either directly or through the intermediacy of a 2-hydroxyisoflavanone, can be used to introduce the isoflavonoid pathway into any plant species that does not naturally possess this pathway. Soybean CYP93C1v2 acts on the flavanones liquiritigenin to yield daidzein, and naringenin to yield genistein. Liquiritigenin is only formed in plants that possess the enzyme chalcone reductase (CHR) (Welle, R. and Grisebach, H., 1989, "Phytoalexin synthesis in soybean cells: elicitor induction of reductase involved in biosynthesis of 6'-deoxychalcone." *Arch Biochem Biophys* 272: 97-102), and a form of chalcone isomerase that is active against 2',4,4'-trihydroxychalcone, the product of the co-action of chalcone synthase (CHS) with CHR (Dixon, R.A., Blyden, E.R., Robbins, M.P., van Tunen, A.J. and Mol, J.N.M., 1988, "Comparative biochemistry of chalcone isomerases." *Phytochemistry* 27: 2801-2808). Such genes are common in legumes, but not in most other plant families. Thus, to form daidzein in transgenic plants that do not possess the isoflavonoid pathway, it would be necessary to introduce three new genes, namely CHR, to co-act with CHS to form 2',4,4'-trihydroxychalcone, a suitable CHI to convert 2',4,4'-trihydroxychalcone to liquiritigenin, and IFS, assuming that the 2-hydroxyisoflavanone intermediate can spontaneously dehydrate in planta, a phenomenon that is demonstrated below. Without CHR present, no liquiritigenin would be formed, and IFS would only be able to act on naringenin to yield, assuming spontaneous dehydration of the 2-hydroxyisoflavanone, genistein.

The IFS genes of the present invention can be introduced into non-leguminous plants such as by standard *Agrobacterium*-based or biolistic transformation procedures (Horsch, et al., 1985, "A simple and general method for transferring genes into plants," *Science* 227:1229-1231; and Klein, et al., 1988, "Stable genetic transformation of intact *Nicotiana* cells by the particle bombardment process," *Proc Natl Acad Sci USA* 85:8502-8505). Both procedures require the construction of a plasmid vector containing a desirable transcriptional promoter driving expression of the gene of interest (in this case IFS), followed by a transcriptional terminator and a

selectable marker gene for resistance, such as to an antibiotic or a herbicide. The biolistic procedure coats metal particles with plasmid DNA containing the gene of interest and places them on a micro carrier disk. Using the biolistic apparatus, the particles are physically propelled into plant tissue. The plant tissue is then put under selection (e.g., antibiotic or herbicide) followed by regeneration. The two *Agrobacterium*-based procedures are "in planta" and "ex-planta", respectively. Both procedures require the above gene construct to be placed into a T-DNA vector, which is then transferred into *Agrobactrium tumefaciens*. The in planta procedure places the transformed *Agrobacterium* in the presence of plant material (flower or meristem) and the plants are allowed to seed followed by selection (e.g., antibiotic or herbicide) during germination. The ex-planta procedure also places *Agrobacterium* in the presence of plant material (callus, cell culture, leaf disk, hypocotyl) which is placed directly under selection (e.g., antibiotic or herbicide) followed by regeneration.

Thus, the isoflavonoid pathway can be introduced into any plant species that does not possess the enzyme catalyzing the IFS reaction by expressing the IFS gene in transgenic plants under the control of a suitable constitutive or inducible promoter.

Example 1: Transformation of *Arabidopsis thaliana* with Soybean *CYP93C1V2*

Soybean CYP93C1v2 cDNA was placed in the binary plant transformation vector pCHF3, in which it is under control of the cauliflower mosaic virus 35S promoter, using standard recombinant DNA methods (Sambrook, et al. 1989. *Molecular Cloning. A Laboratory Manual*, 2nd Ed, Cold Spring Harbor Laboratory Press, New York). The gene was then transformed into plants of the crucifer, *Arabidopsis thaliana* ecotype Columbia, using *Agrobacterium tumefaciens* and a standard floral infiltration procedure (Clough, S.J. and Bent, A.F., 1998, "Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*". *Plant J* 16: 735-743). Transgenic plants were selected by germinating the seedlings on kanamycin, and those surviving selection were allowed to set seed. T_2 seedlings expressing CYP93C1v2 were identified by standard DNA and RNA gel blot analysis (Sambrook, et al. 1989. *Molecular cloning. A Laboratory Manual*, 2nd Ed,

Cold Spring Harbor Laboratory Press, New York), and analyzed for accumulation of genistein in leaves by HPLC analysis, according to a method developed to profile the flavonoid components of *Arabidopsis* leaves (Graham, T.L., 1998, "Flavonoid and flavonol glycoside metabolism in *Arabidopsis*". *Plant Physiol Biochem* 36: 135-144).

Figure 6A shows a typical HPLC trace of a leaf extract from an untransformed plant. The major components are glycosides (containing glucose and rhamnose) of the flavonols kaempferol and quercetin. Plants harboring the soybean CYP93C1v2 gene showed an additional three peaks on HPLC analysis (Fig. 6B), indicated by the arrows labeled as "1," "2" and "3." No free genistein, free 2-hydroxyisoflavanone or 2-hydroxyisoflavanone conjugates were observed. However, following treatment of extracts with almond β-glucosidase (Fig 7B), one of the new peaks disappeared, and free genistein was now observed, consistent with the peak being a glucoside of genistein. LC-MS analysis confirmed the identities of the new compounds as a glucoside of genistein, glucose-rhamnose-genistein, and rhamnose-genistein (Figs. 6C and 7C and 7D, insets). Therefore, expression of CYP93C1v2 in transgenic *Arabidopsis* leads to formation of genistein with no requirement for an enzyme to catalyze the dehydration of the presumed 2-hydroxyisoflavanone intermediate. *Arabidopsis* plants then modify the genistein by exactly the same chemistry they use to conjugate their endogenous flavonols, namely by conjugation to glucose and rhamnose. Transgenic production of conjugates of genistein are suitable for nutraceutical applications, because genistein is also glycosylated in soybean, its natural dietary source (Graham, T.L., 1991, " Flavonoid and isoflavonoid distribution in developing soybean seedling tissues and in seed and root exudates." *Plant Physiol* 95: 594-603).

In addition to introducing the isoflavonoid pathway into plants that do not possess this pathway, the level of isoflavonoid compounds can be controlled in plants that do possess the pathway by manipulating the level of expression of the IFS gene. Increasing the levels of isoflavonoid compounds in leguminous plants by expression of the IFS gene of the present invention in transgenic plants under the control of a suitable constitutive or inducible promoter can be accomplished by standard methods

such as *Agrobacterium*-based or biolistic transformation methods known in the art. Alternatively, the level of isoflavonoid compounds in plants can be reduced by expression of antisense constructs or constructs designed to promote gene silencing that contain an intact IFS gene, or segments thereof, in transgenic plants using methods known in the art. (Bourque, J.E., 1995, "Antisense strategies for genetic manipulations in plants," *Plant Science* 105:125-149; and Angell, S. M. and D. C. Baulcombe, 1997, "Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA," *EMBO J* 16:3675-3684). Antisense constructs for gene silencing are constructed by placing the whole or part of the cDNA in a three prime to five prime orientation behind a desirable transcriptional promoter and ahead of a transcriptional terminator in a plasmid vector. The vector may be used for biolistic transformation or the new antisense gene may be transferred to a T-DNA vector for *Agrobacterium*-based transformation. The actual mechanism of silencing by antisense constructs is unknown. Homology-dependent gene silencing or co-suppression requires the over-expression of a homologous gene; therefore, to achieve co-suppression a construct is made using a strong promoter, the gene of interest (in this case IFS) and a transcriptional terminator. The gene should be transferred to plants as described above. Gene silencing is an epigenetic phenomenon that may or may not occur with a particular gene construct. When it does occur, the inhibition of gene expression can be greater than with the antisense approach.

Isoflavones can be synthesized from flavanones, utilizing recombinant IFS expressed in any suitable bacterial, fungal, algal, or insect cell system. For example, naringenin is extracted in large amounts from grapefruits. A CYP93C1 enzyme can be used convert naringenin to 2,5,7,4'-tetrahydroxyisoflavanone, which spontaneously converts to the valuable nutraceutical genistein under weak acid conditions. Furthermore, daidzin can be synthesized from liquiritigenin utilizing recombinant CYP93C1 and an isoflavone glucosyltransferase (Köster, J. and W. Barz, 1981, "UDP-Glucose: isoflavone 7-*O*-glucosyltransferase from roots of chick pea (*Cicer arietinum* L.)." *Arch Biochem Biophys* 212: 98-104).

Example 2: Methodology Used to Isolate and Identify IFS cDNA Clones

In an attempt to obtain cDNA clones encoding IFS, a functional genomics approach was followed. IFS activity is present in soybean seeds, which accumulate daidzein and genistein. Furthermore, IFS activity can be induced in soybean tissues in response to infection with *Phytophthora infestans*, associated with the accumulation of the isoflavonoid phytoalexin glyceollin (Bhattacharyya, M. K. and E. W. B. Ward, 1987, "Biosynthesis and metabolism of glyceollin I in soybean hypocotyls following wounding or inoculation with *Phytophthora megasperma* f. sp. *glycinea*," *Physiol Mol Plant Path* 31: 387-405). It was also known that an enzyme catalyzing a similar reaction to IFS, namely the 2-hydroxylation of flavanone but without aryl migration, belongs to the CYP93B1 subclass of cytochrome P450s (Akashi, et al, 1998, "Identification of a cytochrome P450 cDNA encoding (2S)-flavanone 2-hydroxylase of licorice (*Glycyrrhiza echinata* L.: *Fabaceae*) which represents licodione synthase and flavone synthase II," *FEBS Letters* 431: 287-290). We therefore searched an expressed sequence tag (EST) database of partial soybean sequences obtained by mass sequencing of two cDNA libraries: a *Phytophthora*-infected hypocotyl cDNA library (48 hours after infection) and a mid to late developmental stage seed library. Nine candidate P450 sequences were identified, of which three belonged to the CYP93 family. DNA probes were made from the EST clones of the three CYP93 candidates and were used to probe an RNA blot of transcripts from alfalfa suspension cells at various times after exposure to yeast elicitor, a treatment known to induce IFS activity at the onset of isoflavonoid phytoalexin accumulation (Kessmann, et al., 1990, "Stress responses in alfalfa (*Medicago sativa* L.) III. Induction of medicarpin and cytochrome P450 enzyme activities in elicitor-treated cell suspension cultures and protoplasts," *Plant Cell Reports* 9: 38-41). One P450 probe cross-hybridized and detected alfalfa transcripts that were strongly induced by elicitation. This probe was derived from a clone with high homology to soybean CYP93C1 as described below, and the insert in the EST clone was full length. The insert was excised and then cloned into the baculovirus expression system for functional identification by heterologous expression in insect cells (Pauli, H. H. and T. M. Kutchan, 1998, "Molecular cloning and functional heterologous expression of two alleles encoding (S)-*N*-methylcoclaurine 3'-

hydroxylase (CYP80B1), a new methyl jasmonate-inducible cytochrome P-450-dependent mono-oxygenase of benzylisoquinoline alkaloid biosynthesis," *The Plant J* 13: 793-801).

The carbon monoxide difference spectrum of microsomes isolated from insect cells expressing the soybean CYP93C clone indicated the presence of expressed cytochrome P450, as seen from an absorption peak at 450 nm that was not present in similar spectra from insect microsomes originating from cells transformed with a control vector. Unlabeled liquiritigenin was then fed to the microsomes in the presence of NADPH. The substrate remained unconverted in microsomes from cells harboring the control vector. However, in microsomes expressing the CYP93C clone, a new peak of RT 29.96 min was observed by high performance liquid chromatography (Fig 8A). The amount of this peak was reduced 10-fold if NADPH was omitted from the incubations (Fig. 8B). The UV spectrum of the product, obtained by diode array detection, was identical to that of authentic daidzein (λmax 248 nm, sh 302 nm, λmin 222 nm). The product was collected, derivatized, and analyzed by GC-MS. The mass spectrum of the BSTFA derivative was identical to that of an authentic sample of daidzein (Fig. 9). Microsomes containing the CYP93C clone also metabolized naringenin to yield genistein, although somewhat less efficiently than the reaction with liquiritigenin (Fig. 8C). Insect cell microsomes expressing a different soybean cytochrome P450 cDNA, CYP93E, did not convert liquiritigenin to daidzein when incubated in the presence of NADPH (Fig. 8D). These results indicate that the soybean CYP93C encodes IFS.

Example 3: Method of Increasing Dietary Isoflavonoid Intake

Transgenic tomato plants are produced by the introduction of *CYP93C1v2* via standard *Agrobacterium*-based procedures. In a preferred embodiment, the CYP93C1v2 coding sequence is under control of a gene promoter giving specific expression in the fruit. Progeny containing the coding region of the *CYP93C1v2* gene are selected at the seedling stage by standard polymerase chain reaction and/or DNA blot analysis known to those skilled in the art. Plants scoring positive for possession of the transgene are grown to fruiting, and fruit analyzed for the presence of

isoflavones by the HPLC methods shown in Fig. 7 and Fig. 8 of the present invention. Fruit harvested from the transgenic tomato plants are ingested to increase the dietary intake of isoflavonoids.

It is to be understood that the above description is of preferred exemplary embodiments of the invention and is intended to be illustrative of the invention, but is not to be construed to limit the scope of the invention in any way. Modifications may be made in the structural features of the invention without departing from the scope of the invention.

In summary, isoflavones can now be genetically engineered to provide potential human health benefits of dietary isoflavones and to increase disease resistance in plants. Isoflavones can now be produced in transgenic plants species in which isoflavones do not naturally occur, i.e., in species other than legumes. For example, engineering constitutive production of daidzein and/or genistein or their conjugates into tomato, potato, corn, or other popular components of the human diet, leads to human health benefits, such as reduced cancer risk, reduced incidence of osteoporosis, and treatment for alcoholism. Alternatively, introducing infection-inducible isoflavonoid biosynthesis into non-legumes qualitatively complements these plants' phytoalexin defenses against microbial pathogens, whereas over-expression of the isoflavonoid pathway in legumes quantitatively increases this defense response. Finally, modifying the extent of production of isoflavonoids in legume roots positively impacts nodulation efficiency and therefore plant yield.

EDITORIAL NOTE

APPLICATION NUMBER - 37287/00

The following Sequence Listing pages 1 to 11 are part of the description. The claims pages follow on pages "29" to "37".

SEQUENCE LISTING

<110> THE SAMUEL ROBERTS NOBLE FOUNDATION, INC.
STEELE, Christopher L.
DIXON, Richard A.

<120> GENETIC MANIPULATION OF ISOFLAVONOIDS

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<151> 1999-03-08

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Lys Val Ser Met Glu Glu Arg Pro Gly Leu Thr Val Pro Arg Ala His
        490                 495                 500

aat ctc atg tgt gtt cct ctt gca aga gct ggt gtc gca gat aaa ctt    1648
Asn Leu Met Cys Val Pro Leu Ala Arg Ala Gly Val Ala Asp Lys Leu
    505                 510                 515

ctt tcc tcc taaaatatct tgagaggatg aatcaccaac atatagcctc            1697
Leu Ser Ser
520

tctttggtac tacaaaatta tgatgtaatt ttcttatttt ttctgtcaca aaggaagtgt 1757

tgtaacttgt aattgcatac aaaatctata aattttatca tcctattcat tatt       1811
```

<210> 5
<211> 522
<212> PRT
<213> Medicago truncatula

<400> 5
Met Leu Val Glu Leu Ala Val Thr Leu Leu Leu Ile Ala Leu Phe Leu
1 5 10 15

His Leu Arg Pro Thr Pro Thr Ala Lys Ser Lys Ala Leu Arg His Leu
20 25 30

Pro Asn Pro Pro Ser Pro Lys Pro Arg Leu Pro Phe Ile Gly His Leu
35 40 45

His Leu Leu Asp Asn Pro Leu Leu His His Thr Leu Ile Lys Leu Gly
50 55 60

Lys Arg Tyr Gly Pro Leu Tyr Thr Leu Tyr Phe Gly Ser Met Pro Thr
65 70 75 80

Val Val Ala Ser Thr Pro Asp Leu Phe Lys Leu Phe Leu Gln Thr His
85 90 95

Glu Ala Thr Ser Phe Asn Thr Arg Phe Gln Thr Ser Ala Ile Ser Arg
100 105 110

Leu Thr Tyr Asp Asn Ser Val Ala Met Val Pro Phe Ala Pro Tyr Trp
115 120 125

Lys Phe Ile Arg Lys Leu Ile Met Asn Asp Leu Leu Asn Ala Thr Thr
130 135 140

Val Asn Lys Leu Arg Pro Leu Arg Ser Arg Glu Ile Leu Lys Val Leu
145 150 155 160

Lys Val Met Ala Asn Ser Ala Glu Thr Gln Gln Pro Leu Asp Val Thr
165 170 175

Glu Glu Leu Leu Lys Trp Thr Asn Ser Thr Ile Ser Thr Met Met Leu
180 185 190

Gly Glu Ala Glu Glu Val Arg Asp Ile Ala Arg Asp Val Leu Lys Ile
195 200 205

Phe Gly Glu Tyr Ser Val Thr Asn Phe Ile Trp Pro Leu Asn Lys Phe
210 215 220

Lys Phe Gly Asn Tyr Asp Lys Arg Thr Glu Glu Ile Phe Asn Lys Tyr
225 230 235 240

Asp Pro Ile Ile Glu Lys Val Ile Lys Lys Arg Gln Glu Ile Val Asn
245 250 255

Lys Arg Lys Asn Gly Glu Ile Val Glu Gly Glu Gln Asn Val Val Phe
260 265 270

Leu Asp Thr Leu Leu Glu Phe Ala Gln Asp Glu Thr Met Glu Ile Lys
275 280 285

```
Ile Thr Lys Glu Gln Ile Lys Gly Leu Val Val Asp Phe Phe Ser Ala
    290                 295                 300

Gly Thr Asp Ser Thr Ala Val Ser Thr Glu Trp Thr Leu Ser Glu Leu
305                 310                 315                 320

Ile Asn Asn Pro Arg Val Leu Lys Lys Ala Arg Glu Glu Ile Asp Ser
                325                 330                 335

Val Val Gly Lys Asp Arg Leu Val Asp Glu Ser Asp Val Gln Asn Leu
            340                 345                 350

Pro Tyr Ile Lys Ala Ile Val Lys Glu Ala Phe Arg Leu His Pro Pro
        355                 360                 365

Leu Pro Val Val Lys Arg Lys Cys Thr Gln Glu Cys Glu Ile Asp Gly
    370                 375                 380

Tyr Val Val Pro Glu Gly Ala Leu Ile Leu Phe Asn Val Trp Ala Val
385                 390                 395                 400

Gly Arg Asp Pro Lys Tyr Trp Val Lys Pro Leu Glu Phe Arg Pro Glu
                405                 410                 415

Arg Phe Ile Glu Asn Val Gly Glu Gly Glu Ala Ala Ser Ile Asp Leu
            420                 425                 430

Arg Gly Gln His Phe Thr Leu Leu Pro Phe Gly Ser Gly Arg Arg Met
        435                 440                 445

Cys Pro Gly Val Asn Leu Ala Thr Ala Gly Met Ala Thr Met Ile Ala
    450                 455                 460

Ser Ile Ile Gln Cys Phe Asp Leu Gln Val Pro Gly Gln His Gly Glu
465                 470                 475                 480

Ile Leu Asn Gly Asp Tyr Ala Lys Val Ser Met Glu Glu Arg Pro Gly
                485                 490                 495

Leu Thr Val Pro Arg Ala His Asn Leu Met Cys Val Pro Leu Ala Arg
            500                 505                 510

Ala Gly Val Ala Asp Lys Leu Leu Ser Ser
        515                 520
```

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for introducing into a naturally non-isoflavonoid-producing plant species the enzyme catalyzing the aryl migration of a flavanone to form an isoflavanone intermediate or an isoflavone, comprising: introducing a DNA segment encoding said enzyme into said plant to form a transgenic plant, wherein said transgenic plant expresses said DNA segment under the control of a suitable constitutive or inducible promoter when said transgenic plant is exposed to conditions which permit expression.

2. The method of claim 1, wherein chalcone synthase, chalcone reductase, and chalcone isomerase genes are also expressed in said plant to cause *in vivo* formation of daidzein or a daidzein derivative.

3. The method of claim 2, wherein said plant is further transformed to comprise said chalcone synthase, chalcone reductase, and chalcone isomerase genes.

4. The method of claim 1 or 2, wherein said plant further comprises downstream genes to metabolize said formed isoflavanone intermediate or isoflavone to biologically active isoflavonoid derivatives or conjugates.

5. The method of claim 4, wherein said downstream gene is selected from the group consisting of isoflavone *O*-methyltransferase, isoflavone 2'-hydroxylase, isoflavone reductase, and vestitone reductase.

6. The method of claim 5, wherein said plant comprises downstream gene 4'-*O*-methyltransferase to form biochanin A or a biochanin A derivative.

7. A method for increasing the level of isoflavonoid compounds in naturally isoflavonoid-producing plants comprising: introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to yield an isoflavonoid to form a transgenic plant, wherein said transgenic plant expresses said DNA segment under the control of a suitable constitutive or inducible promoter when said transgenic plant is exposed to conditions which permit expression.

8. The method of claim 7, wherein said isoflavonoid is selected from the group consisting of an isoflavonone intermediate, an isoflavone, an isoflavone derivative, and an isoflavone conjugate.

9. The method of any preceding claim, wherein said DNA segment comprises isolated genomic DNA.

10. The method of any one of claims 1 to 8, wherein said DNA segment comprises recombinant cDNA.

11. The method of any one of claims 7 to 10, wherein said DNA segment comprises CYP93C gene.

12. The method of any one of claims 7 to 10, wherein said DNA segment is a *Medicago truncatula* homolog of a CYP93C gene.

13. The method of any one of claims 1 to 12, wherein said flavanone is liquiritigenin.

14. The method of any one of claims 1 to 12, wherein said flavanone is naringenin.

15. A method for synthesizing an isoflavanone intermediate or an isoflavone from a flavanone by expressing a recombinant CYP93C gene segment in a suitable bacterial, fungal, algal, or insect cell system.

16. A method of reducing the levels of isoflavonoid compounds in a naturally isoflavonoid-producing plant comprising introducing and expressing an antisense or gene silencing construct that contains an intact CYP93C gene or segments thereof into said plant.

17. The method of any one of claims 1, 11, 15 or 16, wherein said gene comprises a nucleotide sequence encoding the polypeptide of SEQ ID NO:2.

18. The method of any one of claims 1, 12, 15 or 16, wherein said gene comprises a nucleotide sequence encoding the polypeptide of SEQ ID NO:5.

19. A naturally non-isoflavonoid producing plant cell transformed by introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to form an isoflavanone intermediate or an isoflavone, wherein said transgenic plant cell expresses said DNA segment under the control of a suitable constitutive or inducible promoter when exposed to conditions which permit expression.

20. The plant cell of claim 19, wherein chalcone synthase, chalcone reductase, and chalcone isomerase genes are also expressed in said plant to cause *in vivo* formation of daidzein or a daidzein derivative.

21. The plant cell of claim 20, wherein said plant cell is further transformed to comprise said chalcone synthase, chalcone reductase, and chalcone isornerase genes.

22. The plant cell of claim 19 or claim 20, wherein said plant cell further comprises downstream genes to metabolize said formed isoflavanone intermediate or isoflavone to biologically active isoflavonoid derivatives or conjugates.

23. The plant cell of claim 22, wherein said downstream gene is selected from the group consisting of isoflavone *O*-methyltransferase, isoflavone 2'-hydroxylase, isoflavone reductase, and vestitone reductase.

24. The plant cell of claim 23, wherein said plant cell comprises downstream gene 4'-*O*-methyltransferase to form biochanin A or a biochanin A derivative.

25. A naturally isoflavonoid-producing plant cell transformed by introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to yield an isoflavonoid to form a transformed plant cell, wherein said transformed plant cell expresses said DNA segment under the control of a suitable constitutive or inducible promoter when exposed to conditions which permit expression.

26. The plant cell of claim 25, wherein said isoflavonoid is selected from the group consisting of an isoflavonone intermediate, an isoflavone, an isoflavone derivative, and an isoflavone conjugate.

27. The plant cell of any one of claims 19, 25 or 26, wherein said DNA segment comprises isolated genomic DNA.

28. The plant cell of any one of claims 19, 25 or 26, wherein said DNA segment comprises recombinant cDNA.

29. The plant cell of any one of claims 19 or 25 to 28, wherein said DNA segment comprises CYP93C gene.

30. The plant cell of claim 19 or 25-28, wherein said DNA segment is a *Medicago truncatula* homolog of a CYP93C gene.

31. A transgenic plant cell having reduced levels of isoflavonoid compounds, said plant cell transformed by introducing an antisense or gene silencing construct that contains an intact CYP93C gene or segments thereof into said plant cell.

32. The plant cell of claim 29 or claim 31, wherein said gene comprises a nucleotide sequence encoding the polypeptide of SEQ ID NO:2.

33. The plant cell of claim 30 or claim 31, wherein said gene comprises a nucleotide sequence encoding the polypeptide of SEQ ID NO:5.

34. An isolated gene or DNA segment comprising a portion which encodes a cytochrome P450 of the CYP93 family that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion comprises a nucleotide sequence encoding the polypeptide of SEQ ID NO:2.

35. The gene or DNA segment of claim 34, wherein said gene is the soybean gene encoding the enzyme catalyzing the aryl migration of liquiritigenin.

36. The gene or DNA segment of claim 34, wherein said gene is the soybean gene encoding the enzyme catalyzing the aryl migration of naringenin.

37. A protein encoded by a portion of an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone

to yield an isoflavanone intermediate or an isoflavone, wherein said portion comprises a nucleotide sequence encoding the polypeptide of SEQ ID NO:2.

38. An isolated gene or DNA segment comprising a portion which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion is a *Medicago truncatula* homolog of a CYP93C gene.

39. The gene or DNA segment of claim 38 comprising a nucleotide sequence encoding the polypeptide of SEQ ID NO:5.

40. The gene or DNA segment of claim 38 or claim 39, wherein said gene is the *Medicago truncatula* gene encoding the enzyme catalyzing the aryl migration of liquiritigenin.

41. The gene or DNA segment of claim 38 or claim 39, wherein said gene is the *Medicago truncatula* gene encoding the enzyme catalyzing the aryl migration of naringenin.

42. A protein encoded by a portion of an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion is a *Medicago truncatula* homolog of a CYP93C gene.

43. A transgenic plant cell transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant cell exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plant cells of the same species which do not comprise said isolated gene or DNA segment.

44. A food comprising edible transgenic plant material capable of being ingested for its nutritional value, wherein said transgenic plant comprises plant cells according to claim 43.

45. A method of preparing a food comprising at least one isoflavonoid comprising: transforming a plant according to the method of any one of claims 1 to 12, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said DNA segment, isolating said isoflavonoid and incorporating into said food.

46. A composition comprising at least a portion of a transgenic plant according to claim 43, wherein said composition is suitable for ingestion as a food stuff, a nutritional supplement, an animal feed supplement, or a nutraceutical.

47. A method of preparing a composition comprising an isoflavonoid suitable for administration as a food stuff, a nutritional supplement, an animal feed supplement, a nutraceutical, or a pharmaceutical, comprising: transforming a plant according to the method of any one of claims 1 to 12, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said DNA segment, isolating said isoflavonoid and incorporating into said compositions.

48. A method of using a transgenic plant according to claim 43 to provide a nutraceutical benefit to a human or animal administered said isoflavonoid.

49. The method of claim 48, wherein said isoflavonoid is administered by ingestion of at least a portion of said plant.

50. The method of claim 48, wherein said isoflavonoid is administered by ingestion of a composition comprising an isoflavonoid isolated from said plant.

51. A method for making a pharmaceutical preparation, comprising: transforming a plant according to the method of any one of claims 1 to 12, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said DNA segment, isolating said isoflavonoid and formulating said isoflavonoid to form a pharmaceutical preparation.

52. A method of transforming a plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone comprising introducing the isolated gene or DNA segment into the plant, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment.

53. The method of claim 52, wherein the nutritional value of said plant is increased.

54. The method of claim 52, wherein the disease resistance in said plant is increased.

55. The method of claim 52, wherein bacterial or fungal symbiosis in said plant is increased.

56. The method of claim 52, wherein said plant is a leguminous plant.

57. The method of claim 56, wherein the nodulation efficiency of said plant is increased.

58. A leguminous transgenic plant exhibiting increased nodulatlon efficiency, wherein said transgenic plant is transformed according to the method of claim 52.

59. The transgenic plant of claim 43 exhibiting an increased level of bacterial or fungal symbiosis.

60. A transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said recombinant DNA sequence.

61. The transgenic plant according to claim 60, wherein the recombinant DNA sequence comprises a nucleotide sequence encoding the polypeptide of SEQ ID NO:2.

62. The transgenic plant according to claim 60, wherein the recombinant DNA sequence comprises a nucleotide sequence encoding the polypeptide of SEQ ID NO:5.

63. Seed from a transgenic plant according to claim 60.

64. Progeny from a transgenic plant according to claim 60.

65. Progeny from seed of a transgenic plant according to claim 60.

66. Use of a transgenic plant according to claim 43 for the preparation of a nutraceutical preparation for achieving a nutritional effect.

67. Use of a transgenic plant according to claim 43 for the preparation of a pharmaceutical preparation for achieving a therapeutic effect.

68. A method according to any one of claims 1, 7, 15, 16, 45, 47, 51 or 52 substantially as hereinbefore described with reference to the examples.

69. A plant cell according to any one of claims 19, 25, 31 or 43 substantially as hereinbefore described with reference to the examples.

70. An isolated gene or DNA segment according to claim 34 or claim 38 substantially as hereinbefore described with reference to the examples.

71. A protein according to claim 42 substantially as hereinbefore described with reference to the examples.

72. A food according to claim 44 substantially as hereinbefore described with reference to the examples.

73. A composition according to claim 46 substantially as hereinbefore described with reference to the examples.

74. A transgenic plant according to claim 58 or claim 60 substantially as hereinbefore described with reference to the examples.

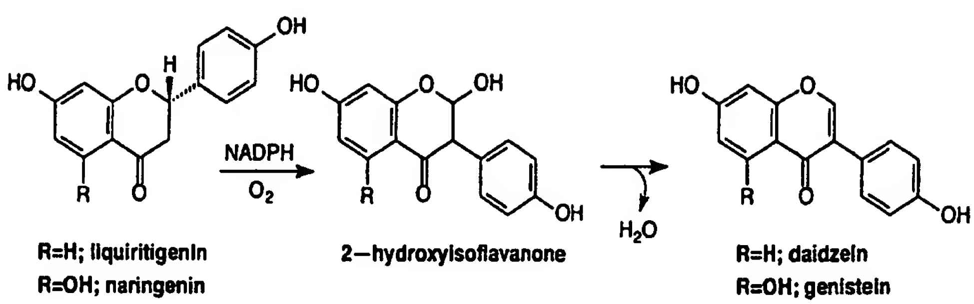
HO
OH
H
NADPH
O_2
R
O
OH
H_2O
R=H; liquiritigenin
R=OH; naringenin
2-hydroxyisoflavanone
R=H; daidzein
R=OH; genistein

Fig. 1

1 GAGCAAAGAT CAAACAAACC AAGGACGAGA ACACGATGTT GCTTGAACTT
51 GCACTTGGTT TATTGGTTTT GGCTCTGTTT CTGCACTTGC GTCCCACACC
101 CACTGCAAAA TCAAAAGCAC TTCGCCATCT CCCAAACCCA CCAAGCCCAA
151 AGCCTCGTCT TCCCTTCATA GGACACCTTC ATCTCTTAAA AGACAAACTT
201 CTCCACTACG CACTCATCGA CCTCTCCAAA AAACATGGTC CCTTATTCTC
251 TCTCTACTTT GGCTCCATGC CAACCGTTGT TGCCTCCACA CCAGAATTGT
301 TCAAGCTCTT CCTCCAAACG CACGAGGCAA CTTCCTTCAA CACAAGGTTC
351 CAAACCTCAG CCATAAGACG CCTCACCTAT GATAGCTCAG TGGCCATGGT
401 TCCCTTCGGA CCTTACTGGA AGTTCGTGAG GAAGCTCATC ATGAACGACC
451 TTCTCAACGC CACCACTGTA AACAAGTTGA GGCCTTTGAG GACCCAACAG
501 ATCCGCAAGT TCCTTAGGGT TATGGCCCAA GGCGCAGAGG CACAGAAGCC
551 CCTTGACTTG ACCGAGGAGC TTCTGAAATG GACCAACAGC ACCATCTCCA
601 TGATGATGCT CGGCGAGGCT GAGGAGATCA GAGACATCGC TCGCGAGGTT
651 CTTAAGATCT TTGGCGAATA CAGCCTCACT GACTTCATCT GGCCATTGAA
701 GCATCTCAAG GTTGGAAAGT ATGAGAAGAG GATCGACGAC ATCTTGAACA
751 AGTTCGACCC TGTCGTTGAA AGGGTCATCA AGAAGCGCCG TGAGATCGTG
801 AGGAGGAGAA AGAACGGAGA GGTTGTTGAG GGTGAGGTCA GCGGGGTTTT
851 CCTTGACACT TTGCTTGAAT TCGCTGAGGA TGAGACCATG GAGATCAAAA
901 TCACCAAGGA CCACATCAAG GGTCTTGTTG TCGACTTTTT CTCGGCAGGA
951 ACAGACTCCA CAGCGGTGGC AACAGAGTGG GCATTGGCAG AACTCATCAA
1001 CAATCCTAAG GTGTTGGAAA AGGCTCGTGA GGAGGTCTAC AGTGTTGTGG
1051 GAAAGGACAG ACTTGTGGAC GAAGTTGACA CTCAAAACCT TCCTTACATT
1101 AGAGCAATCG TGAAGGAGAC ATTCCGCATG CACCCGCCAC TCCCAGTGGT
1151 CAAAAGAAAG TGCACAGAAG AGTGTGAGAT TAATGGATAT GTGATCCCAG
1201 AGGGAGCATT GATTCTCTTC AATGTATGGC AAGTAGGAAG AGACCCCAAA
1251 TACTGGGACA GACCATCGGA GTTCCGTCCT GAGAGGTTCC TAGAGACAGG
1301 GGCTGAAGGG GAAGCAGGGC CTCTTGATCT TAGGGGACAA CATTTTCAAC
1351 TTCTCCCATT TGGGTCTGGG AGGAGAATGT GCCCTGGAGT CAATCTGGCT
1401 ACTTCGGGAA TGGCAACACT TCTTGCATCT CTTATTCAGT GCTTCGACTT
1451 GCAAGTGCTG GGTCCACAAG GACAGATATT GAAGGGTGGT GACGCCAAAG
1501 TTAGCATGGA AGAGAGAGCC GGCCTCACTG TTCCAAGGGC ACATAGTCTT
1551 GTCTGTGTTC CACTTGCAAG GATCGGCGTT GCATCTAAAC TCCTTTCTTA
1601 ATTAAGATCA TCGTCATCAT CATCATATGT AATATTTACT TTTTGTGTGT
1651 TGATAATCAT CATTTCAATA AGGTCTCATT CATCTACTTT TTATGAAGTA
1701 TATAAGCCCT TCCATGC

Fig. 2

```
               .
CYP93C1v2    1 ...MLLELALGLLVLALFLHLRPTPTAKSKALRHLPNPPSPKPRLPFIGH 47
                   |. ... .  |  :   ||      | |     ||||  ||| |||
CYP93B1      1 MEPQLVAVSVLVSALICYFFFRPYFHRYGKNL.....PPSPFFRLPIIGH 45

                        .         .         .         .         .
            48 LHLLKDKLLHYALIDLSKKHGPLFSLYFGSMPTVVASTPELFKLFLQTHE 97
               :|:|   ||| .  .|| ::|||||| |||.  ||||||   |  |||.|
            46 MHML.GPLLHQSFHNLSHRYGPLFSLNFGSVLCVVASTPHFAKQLLQTNE 94

                        .         .         .         .         .
            98 ATSFNTRFQTSAIRRLTYDSSVAMVPFGPYWKFVRKLIMNDLLNATTVNK 147
                 .|| | :..|:::|||:||.|  |:| ||:|::|| ||:|| .  .:|
            95 .LAFNCRIESTAVKKLTYESSLAFAPYGDYWRFIKKLSMNELLGSRSINN 143

                        .         .         .         .         .
           148 LRPLRTQQIRKFLRVMAQGAEAQKPLDLTEELLKWTNSTISMMMLGEAEE 197
                . || |:  . ||.:.  | | . ..:|||||| ||. ||.||.|||||
           144 FQHLRAQETHQLLRLLSNRARAFEAVNITEELLKLTNNVISIMMVGEAEE 193

                        .         .         .         .         .
           198 IRDIAREVLKIFGEYSLTDFIWPLKHLKVGKYEKRIDDILNKFDPVVERV 247
                ||: |:| .||||:...||||  | : .  : |||:|:  :|| .|||:
           194 ARDVVRDVTEIFGEFNVSDFIWLFKKMDLQGFGKRIEDLFQRFDTLVERI 243

                        .         .         .         .         .
           248 IKKRREIVR.RRKNGEVVE.GEVSGV..FLDTLLEFAEDETMEIKITKDH 293
               | || :  : ||:||.  | |   |:  ||| ||:  |||  |||| : |
           244 ISKREQTRKDRRRNGKKGEQGSGDGIRDFLDILLDCTEDENSEIKIQRVH 293

                        .         .         .         .         .
           294 IKGLVVDFFSAGTDSTAVATEWALAELINNPKVLEKAREEVYSVVGKDRL 343
               || |:.|||.||||.||:.||||| ||:  | ||:| |||: .|||||||
           294 IKALIMDFFTAGTDTTAISTEWALVELVKKPSVLQKVREEIDNVVGKDRL 343

                        .         .         .         .         .
           344 VDEVDTQNLPYIRAIVKETFRMHPPLPVVKRKCTEECEINGYVIPEGALI 393
               |:| |  ||||:.||.|||||:|||.|.| |:|  || :  ||||| .|:
           344 VEESDCPNLPYLQAILKETFRLHPPVPMVTRRCVAECTVENYVIPEDSLL 393

                        .         .         .         .         .
           394 LFNVWQVGRDPKYWDRPSEFRPERFLETGAEGEAGPLDLRGQHFQLLPFG 443
                 ||| :||.||:|| | ||||||||.   :  .| .|.|| ||||||||
           394 FVNVWSIGRNPKFWDNPLEFRPERFLKLEGD.SSGVVDVRGSHFQLLPFG 442

                        .         .         .         .         .
           444 SGRRMCPGVNLATSGMATLLASLIQCFDLQVLGPQGQILKGGDAKVSMEE 493
               |||||||||.||   .  || .:|||||  |.||.|:|||| |  :..:|
           443 SGRRMCPGVSLAMQEVPALLGAIIQCFDFHVVGPKGEILKGDDIVINVDE 492

                        .         .         .
           494 RAGLTVPRAHSLVCVPLARIGVASKLLS... 521
               | ||| ||||.|||||. |      |
           493 RPGLTAPRAHNLVCVPVDRTSGGGPLKIIEC 523
```

Fig. 3

1 CAACACCTAA GAGTAACTAA TAAGAACTTT CTTTCTACTT CTTAGTATAC
51 TTAACAACTT AAGTAAATAT ACTACAAAGA AGCTATACAC CATGTTGGTG
101 GAACTTGCAG TTACTCTATT GCTCATTGCT CTCTTCTTAC ACTTGCGTCC
151 AACACCTACT GCAAAATCAA AGGCTCTTCG CCACCTTCCA AATCCACCAA
201 GCCCTAAACC ACGTCTTCCA TTCATAGGTC ATCTTCACCT TTTGGATAAC
251 CCACTTCTTC ACCACACTCT TATCAAGTTA GGAAAGCGTT ATGGCCCTTT
301 GTACACTCTT TACTTTGGTT CCATGCCTAC CGTTGTTGCA TCCACTCCTG
351 ACTTGTTTAA ACTTTTCCTT CAAACCCATG AAGCTACTTC CTTTAACACA
401 AGATTCCAAA CCTCTGCTAT TAGTCGTCTT ACCTATGACA ACTCTGTTGC
451 TATGGTTCCA TTTGCACCTT ATTGGAAGTT TATTAGAAAG CTTATCATGA
501 ACGACTTGCT CAACGCCACC ACTGTTAACA AATTGAGGCC ATTGAGGAGC
551 CGAGAAATCC TTAAGGTTCT TAAGGTCATG GCTAATAGTG CTGAAACTCA
601 ACAGCCACTT GATGTCACTG AGGAGCTTCT CAAGTGGACA AACAGCACAA
651 TCTCTACCAT GATGTTGGGT GAGGCCGAAG AGGTTAGAGA TATTGCTCGT
701 GATGTTCTTA AGATCTTTGG AGAATATAGT GTTACAAACT TTATTTGGCC
751 TTTGAACAAG TTTAAGTTTG GAAACTATGA TAAGAGAACT GAGGAGATTT
801 TCAATAAGTA TGATCCTATC ATTGAAAAGG TTATCAAGAA ACGACAAGAG
851 ATTGTGAACA AAAGAAAAAA TGGAGAAATC GTAGAAGGCG AGCAGAATGT
901 TGTTTTTCTT GACACTTTGC TTGAATTTGC ACAAGATGAG ACCATGGAGA
951 TCAAAATTAC AAAGGAACAA ATCAAGGGTC TTGTTGTGGA TTTTTTCTCT
1001 GCAGGAACAG ACTCCACCGC CGTGTCTACA GAATGGACTT TATCAGAGCT
1051 CATCAATAAT CCTAGAGTGT TGAAGAAAGC TCGAGAGGAG ATTGACTCTG
1101 TTGTGGGAAA AGATAGACTG GTTGATGAAT CAGATGTTCA GAATCTTCCT
1151 TACATTAAAG CCATCGTAAA AGAAGCATTT CGCTTGCACC CACCACTACC
1201 TGTAGTCAAA AGAAAATGTA CACAAGAATG TGAGATCGAC GGGTATGTGG
1251 TTCCAGAAGG AGCACTAATA CTTTTCAATG TCTGGGCAGT GGGAAGAGAC
1301 CCAAAATATT GGGTAAAGCC ATTGGAATTT CGTCCAGAGA GGTTCATAGA
1351 AAATGTTGGT GAAGGTGAAG CAGCTTCAAT TGATCTTAGG GGTCAACATT
1401 TCACACTTCT ACCATTTGGG TCTGGAAGAA GGATGTGTCC TGGAGTCAAT
1451 TTGGCTACTG CAGGAATGGC CACAATGATT GCATCTATTA TCCAATGCTT
1501 CGATCTCCAA GTACCTGGTC AACATGGAGA AATATTGAAT GGTGATTATG
1551 CTAAGGTTAG CATGGAAGAG AGACCTGGTC TCACAGTTCC AAGGGCACAT
1601 AATCTCATGT GTGTTCCTCT TGCAAGAGCT GGTGTCGCAG ATAAACTTCT
1651 TTCCTCCTAA AATATCTTGA GAGGATGAAT CACCAACATA TAGCCTCTCT
1701 TTGGTACTAC AAAATTATGA TGTAATTTTC TTATTTTTTC TGTCACAAAG
1751 GAAGTGTTGT AACTTGTAAT TGCATACAAA ATCTATAAAT TTTATCATCC
1801 TATTCATTAT T

Fig. 4

```
mtIFS       1    MLVELAVTLLLIALFLHLRPTPTAKSKALRHLPNPPSPKPRLPFIGHLHL  50
                 ||.|||. ||.:||||||||||||||||||||||||||||||||||||||
CYP93C1v2   1    MLLELALGLLVLALFLHLRPTPTAKSKALRHLPNPPSPKPRLPFIGHLHL  50
                          .         .         .         .         .
            51   LDNPLLHHTLIKLGKRYGPLYTLYFGSMPTVVASTPDLFKLFLQTHEATS  100
                 | . |||: || | |::|||:.||||||||||||||:|||||||||||||
            51   LKDKLLHYALIDLSKKHGPLFSLYFGSMPTVVASTPELFKLFLQTHEATS  100
                          .         .         .         .         .
            101  FNTRFQTSAISRLTYDNSVAMVPFAPYWKFIRKLIMNDLLNATTVNKLRP  150
                 |||||||||| |||||.||||||| |||||:|||||||||||||||||||
            101  FNTRFQTSAIRRLTYDSSVAMVPFGPYWKFVRKLIMNDLLNATTVNKLRP  150
                          .         .         .         .         .
            151  LRSREILKVLKVMANSAETQQPLDVTEELLKWTNSTISTMMLGEAEEVRD  200
                 ||..:| | |:|||  || |.|||.||||||||||||| ||||||||:||
            151  LRTQQIRKFLRVMAQGAEAQKPLDLTEELLKWTNSTISMMMLGEAEEIRD  200
                          .         .         .         .         .
            201  IARDVLKIFGEYSVTNFIWPLNKFKFGNYDKRTEEIFNKYDPIIEKVIKK  250
                 |||:|||||||||.|.|||||   | | |:|| ::| ||:||::|:||||
            201  IAREVLKIFGEYSLTDFIWPLKHLKVGKYEKRIDDILNKFDPVVERVIKK  250
                          .         .         .         .         .
            251  RQEIVNKRKNGEIVEGEQNVVFLDTLLEFAQDETMEIKITKEQIKGLVVD  300
                 |.||| :|||||:|||| . ||||||||||:||||||||||: |||||||
            251  RREIVRRRKNGEVVEGEVSGVFLDTLLEFAEDETMEIKITKDHIKGLVVD  300
                          .         .         .         .         .
            301  FFSAGTDSTAVSTEWTLSELINNPRVLKKAREEIDSVVGKDRLVDESDVQ  350
                 |||||||||||.||| |.||||||:||.|||||: ||||||||||| | |
            301  FFSAGTDSTAVATEWALAELINNPKVLEKAREEVYSVVGKDRLVDEVDTQ  350
                          .         .         .         .         .
            351  NLPYIKAIVKEAFRLHPPLPVVKRKCTQECEIDGYVVPEGALILFNVWAV  400
                 |||||:||||| ||:||||||||||||:||||.|||:||||||||||| |
            351  NLPYIRAIVKETFRMHPPLPVVKRKCTEECEINGYVIPEGALILFNVWQV  400
                          .         .         .         .         .
            401  GRDPKYWVKPLEFRPERFIENVGEGEAASIDLRGQHFTLLPFGSGRRMCP  450
                 ||||||| :| |||||||:|   ||||  :||||||| ||||||||||||
            401  GRDPKYWDRPSEFRPERFLETGAEGEAGPLDLRGQHFQLLPFGSGRRMCP  450
                          .         .         .         .         .
            451  GVNLATAGMATMIASIIQCFDLQVPGQHGEILNGDYAKVSMEERPGLTVP  500
                 ||||||.||||::||:|||||||| |  |:|| |  |||||||| |||||
            451  GVNLATSGMATLLASLIQCFDLQVLGPQGQILKGGDAKVSMEERAGLTVP  500
                          .         .         .         .         .
            501  RAHNLMCVPLARAGVADKLLSS  522
                 |||.|.|||||| ||| ||||
            501  RAHSLVCVPLARIGVASKLLS   521
                          .         .
```

Fig. 5

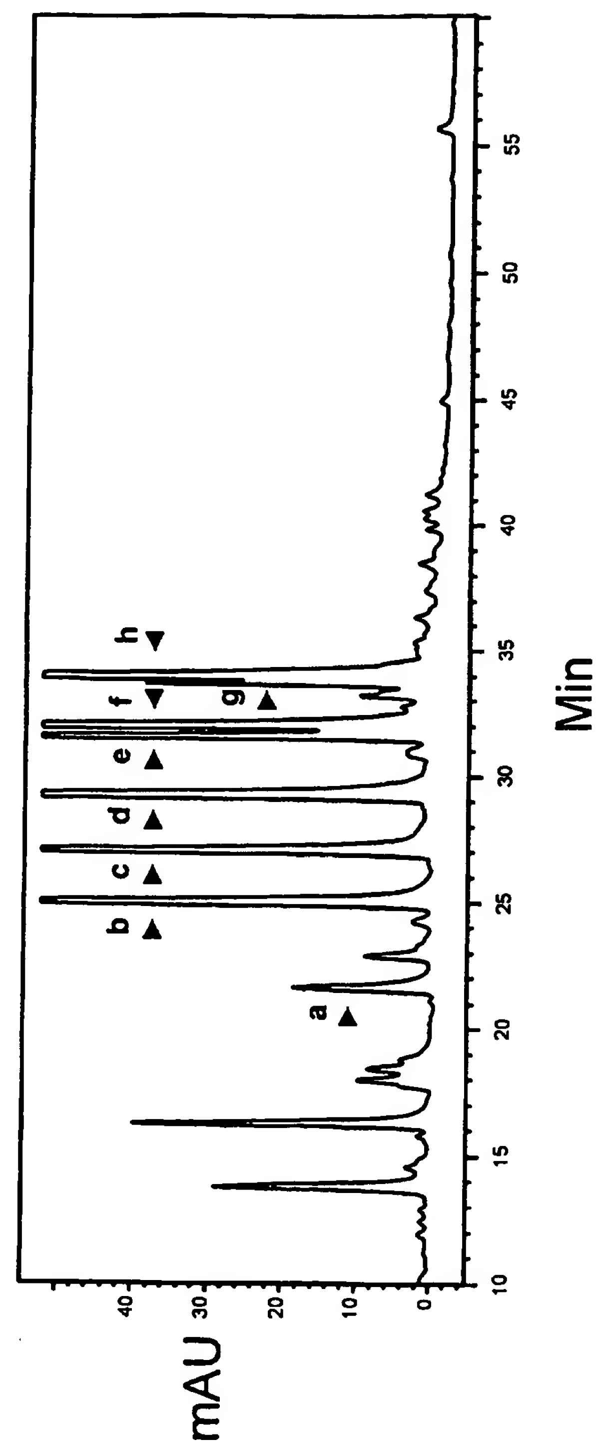

Fig. 6A

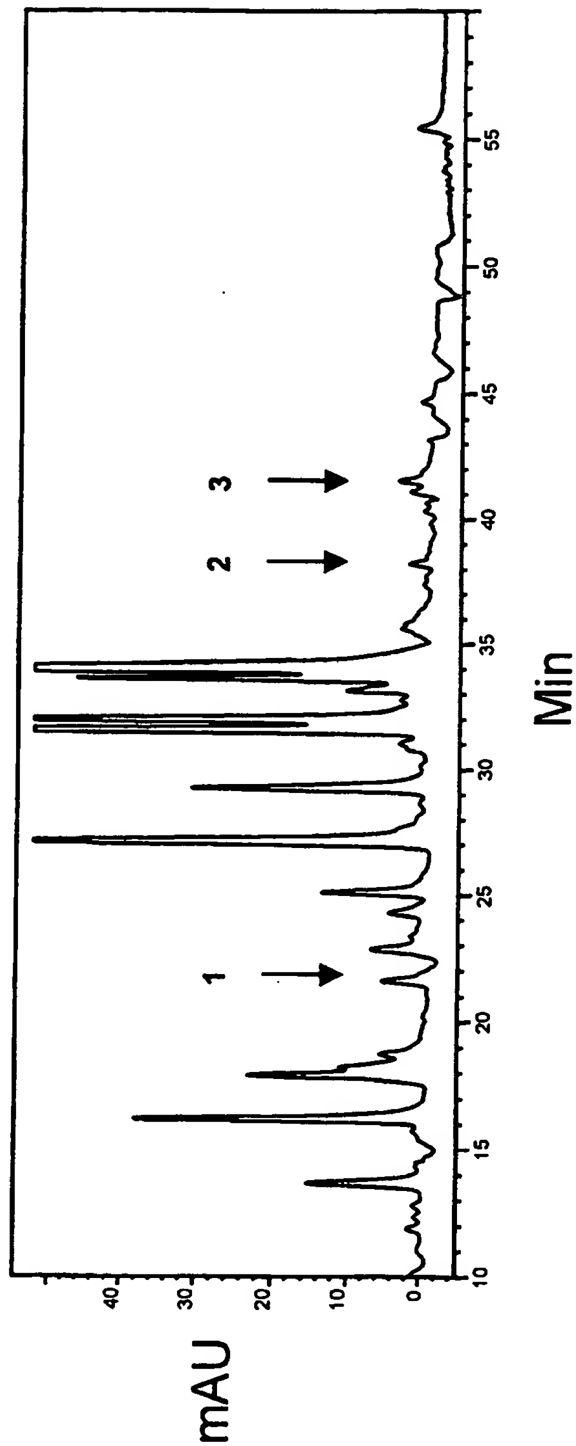

Fig. 6B

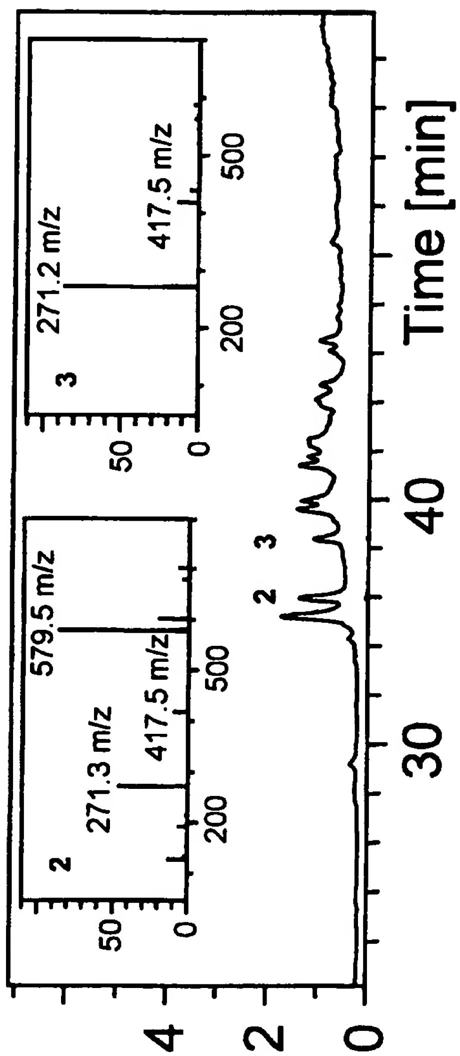
4
2
0
30
40
Time [min]
2
271.3 m/z
417.5 m/z
579.5 m/z
50
0
200
500
3
271.2 m/z
417.5 m/z
50
0
200
500
2
3

Fig. 6C

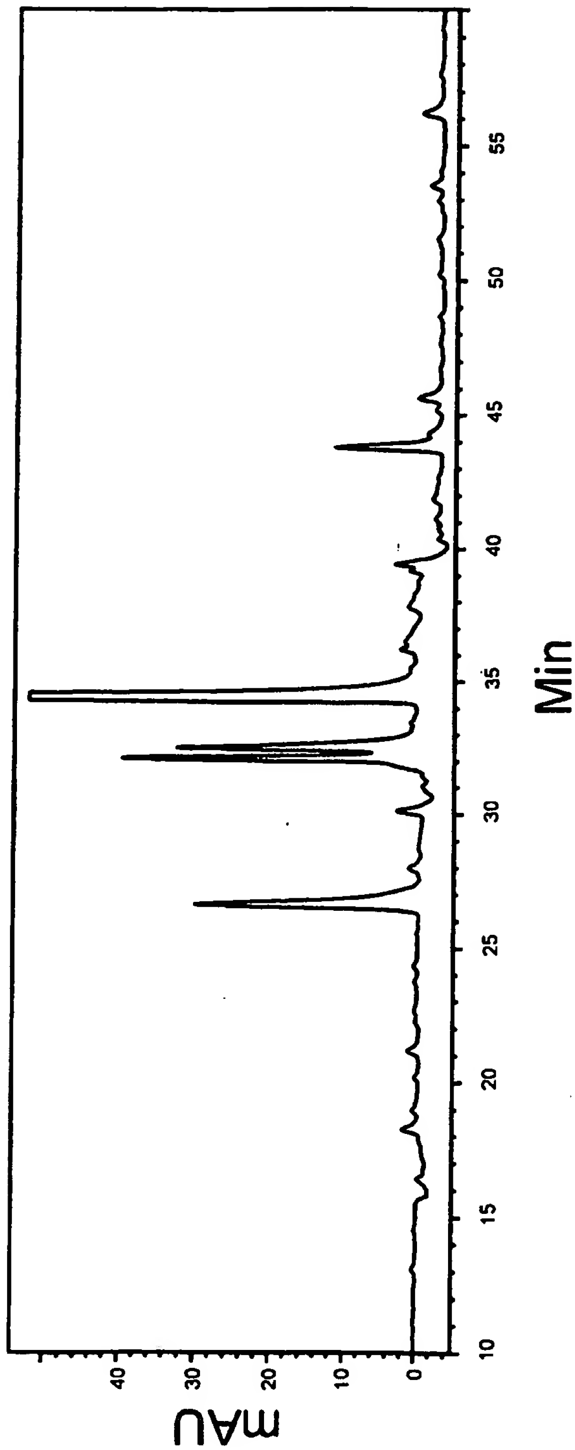

Fig. 7A

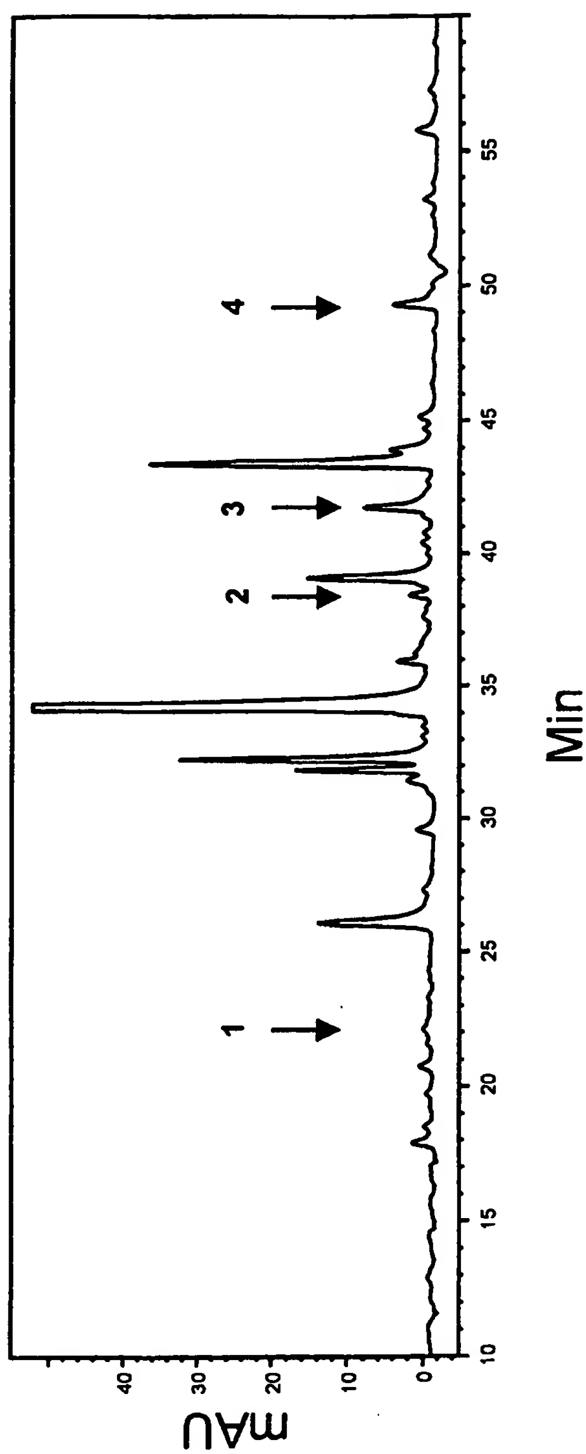

Fig. 7B

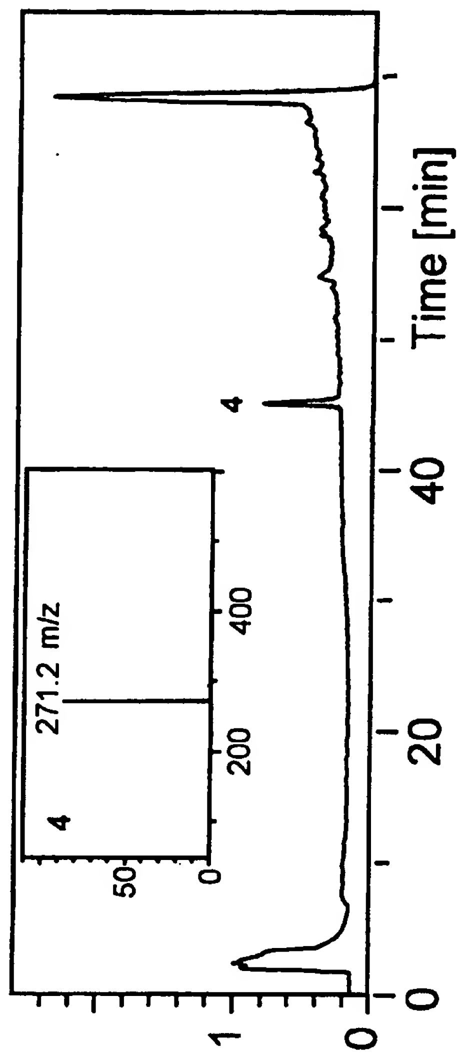

Fig. 7C

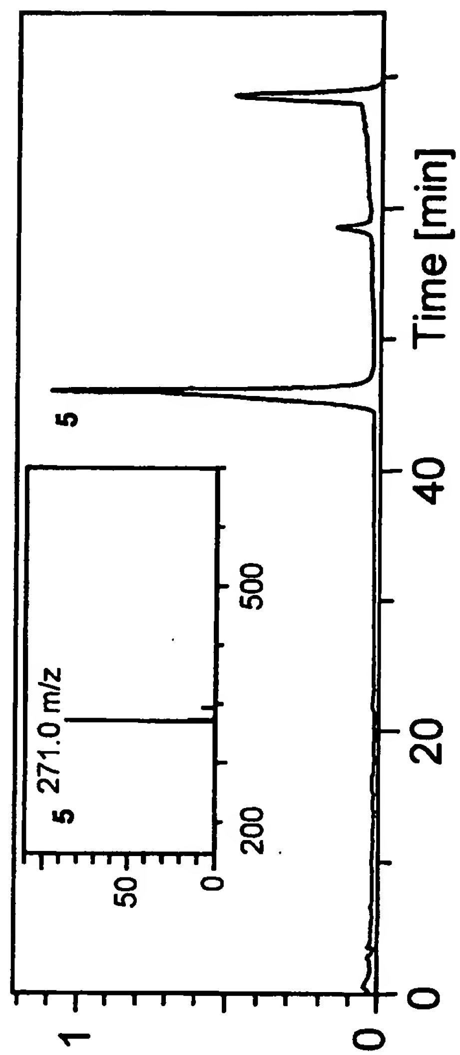

Fig. 7D

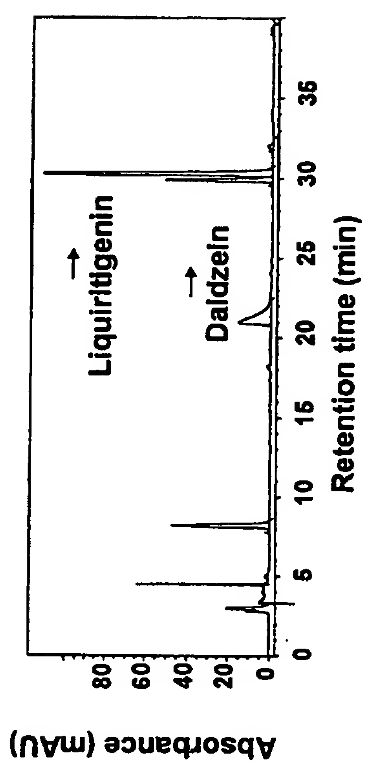
Liquiritigenin
Daidzein
Retention time (min)
Absorbance (mAU)

Fig. 8A

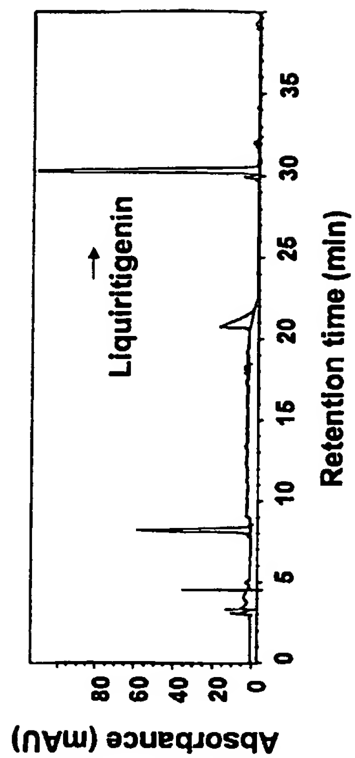
Liquiritigenin
Retention time (min)
Absorbance (mAU)

Fig. 8B

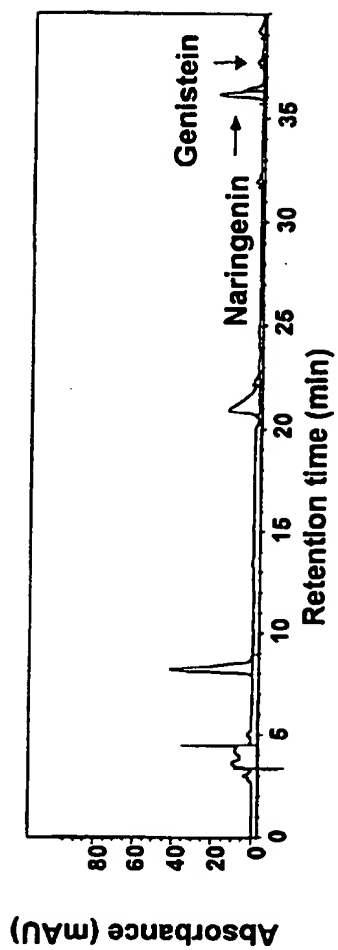
Genistein
Naringenin
Retention time (min)
Absorbance (mAU)
0
5
10
15
20
25
30
35
0
20
40
60
80

Fig. 8C

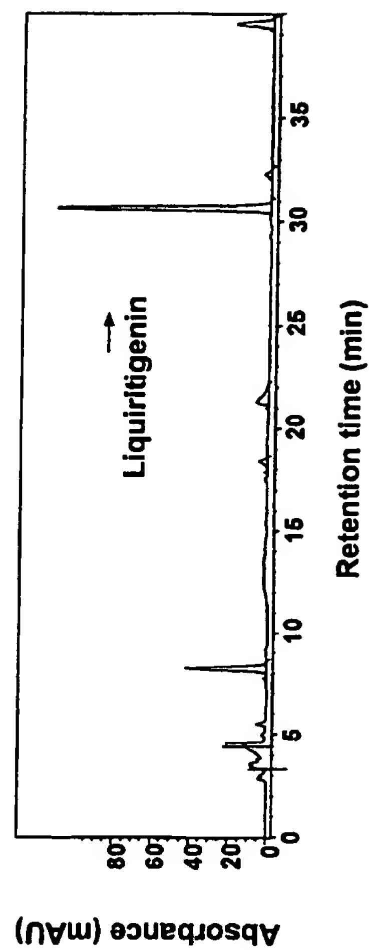
Liquiritigenin
Retention time (min)
Absorbance (mAU)
0
5
10
15
20
25
30
35
0
20
40
60
80

Fig. 8D

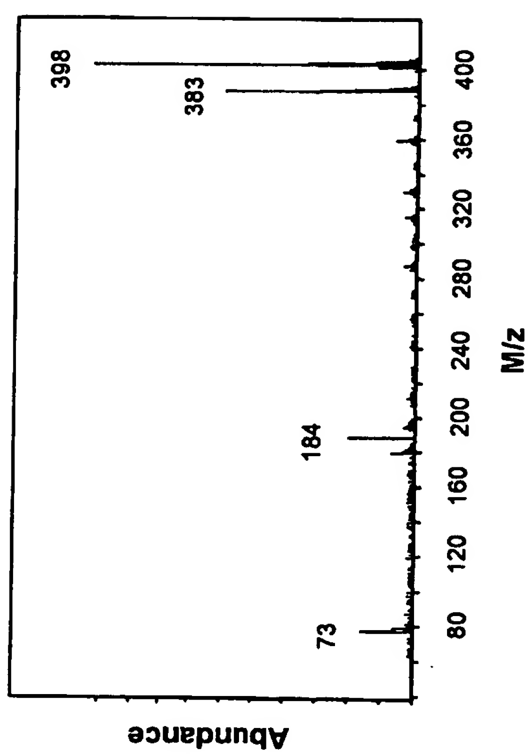
398
383
184
73
80
120
160
200
240
280
320
360
400
M/z
Abundance

Fig. 9A

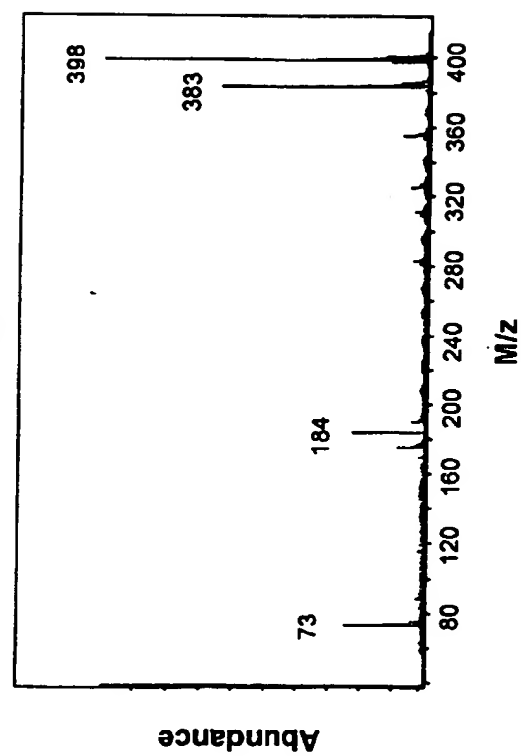
398
383
184
73
80
120
160
200
240
280
320
360
400
M/z
Abundance

Fig. 9B